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Excitability Curves of Physiological and Pathological Dog Auricle in the Intact Animal.* (21525)

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Studies on the refractory period of the auricle have thus far been carried out on exposed hearts. Brooks *et al.*(1,2) recognizing the need to avoid variations in temperature and water and CO_2 loss encased their preparations inside a transparent artificial chest wall. Nevertheless studies on ventricular excitability by this method revealed a type of relative refractory period with "dips" that was not present in the curves obtained from the ventricle in its normal physiological environment(3). The question thus arose whether the excitability of the auricle in its normal environment would be different from that which has been reported on auricles in open chest experiments.

The *method* of Delgado and Sikand(4), which consisted of sewing upon the auricular myocardium, electrodes carried by a polyethylene plate, was employed in this study. The wires attached to the electrode points were carried to the surface through a polyethylene tube and the chest closed in layers after the lungs were expanded. In this way a pneumothorax was avoided and the animals breathed spontaneously. 2-4 hours were allowed to elapse after the operation to permit time for the return of normal physiological conditions. As the experiments proceeded it became necessary to widen the polyethylene plate carrying the electrodes so that the suture points were well removed from the electrodes to allow testing of undamaged muscle. In addition the electrodes themselves were made spherical instead of flat and bent at right angles to project ½ mm below the plates so as to insure contact of the electrodes with the epicardium for a longer time, since it was

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observed that fibrin deposit on the epicardium increased in thickness from day to day and gradually separated the electrodes from the myocardium. It will be seen below that a stronger shock was required each day to produce a response because of this occurrence. In some of the experiments electrodes were sewed to both the right and left auricle to determine whether the excitability varied between the right and left. However no significant differences were found.

In this communication 3 types of excitability curves of the auricles will be described which were obtained by this method.

(a) The normal excitability curve Fig. 1A. It can be seen that an absolute refractory period is followed by a relative refractory period with a smoothly declining slope and a constant diastolic threshold at a level well under 1 m. amp. and averaging 0.6 m. amp. This is typical of 22 experiments performed, 10 on the right and 12 on the left auricle in 11 dogs. In the first 3 animals curves were obtained 2-4 hours after chest closure and again 2-3 hours later. The second determination in each animal was identical with the first one. This indicated that normal conditions had been reestablished 2 hours after chest closure. The hearts of 3 animals were examined within 12 hours after the electrodes were implanted and both the epicardium and myocardium under the electrode points were normal. Because of the uniformity of all the curves and the absence of pathology in the auricles, it was considered that the curves truly represented normal auricular excitability. It will be observed that the excitability of the normal auricle resembled closely the excitability of the normal ventricle with the exception that the duration of the total refractory period of the auricle (170 to 250 msec.) was less than that of the ventricle (200 to 350 msec.).

(b) A curve with the refractory period normal, but with an elevated diastolic threshold (Fig. 1b). Twenty-one such observations, 12 on the right and 11 on the left auricle, were made in 10 animals from 24 to 72 hours post-operative. The diastolic threshold rose progressively from day to day and after 72 hours was usually unobtainable. Parallel with

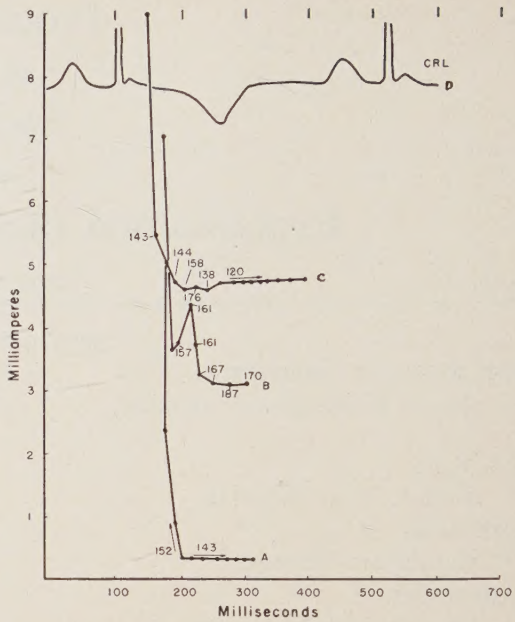

FIG. 1. A. Excitability curve 3 hr after electrode implantation. Abscissa measured from onset of P. Total refractory period is 169 m.sec. and measured from position of ectopic beat in auricular cycle. Note smooth relative refractory period and constant threshold at 0.3 m.amp. Numbers are heart rates at each point and arrows indicate where heart rate remained the same. B. Excitability curve 72 hr after implantation of electrodes. Note oscillation in relative refractory period. Prolongation of total refractory period to 236 m.sec. even though heart rates are faster. C. Same as A but 48 hr later. Note elevation of diastolic threshold to 4.5 m.amp. Total refractory period unchanged. Early threshold period slightly irregular but variation is less than 0.1 m.amp. D. Precordial electrocardiograms taken with exploring electrode over left apex. Indifferent electrode on right forelimb.

the rising diastolic threshold, there developed under the electrode plates a fibrin deposit which grew in thickness and lifted the electrode points away from the muscle. It was considered therefore that the elevated diastolic threshold did not represent a rise in muscle threshold.

(c) A pathological curve which differed from the normal in a prolonged refractory period, the appearance of dips in the relative refractory period as well as an elevated diastolic threshold level, Fig. 1C. This type of curve was observed in 9 animals that were studied on the 2nd to the 4th day post-operative. The animals were sacrificed for histological study at intervals up to 21 days. Sections were

taken underneath the electrode points. Fibrinosis of varying thickness was found on the epicardium less in the earlier than in the later specimens. In addition myocardial pathology was also observed in the form of focal areas of hemorrhagic necrosis. These areas were gradually replaced by fibrosis. A cellular exudate accompanying the necrosis changed gradually from predominantly polymorphonuclear leucocytes to predominantly lymphocytes and large monocytes. Since these lesions developed under the electrode points, they were attributed to trauma of the points against the contracting auricle. It was considered that this type of curve was the result of the myocardial pathology.

This method of testing the excitability of the auricles is obviously limited since only in the first 12 hours after the electrodes are implanted and the chest closed, can one be reasonably confident of obtaining a normal excitability curve. However, it can be employed to study the effect of drugs and other factors on the excitability of the normal auricle if the total duration of the experiment is within 12 hours.

Summary. 1. Three types of excitability curves of the dog auricle are described which were obtained by the method of permanent electrode implantation on either right or left auricle of the dog heart. 2. A normal curve can always be obtained within the first 12 hours after the operation. It consists of an absolute refractory period, a relative refractory period with a smoothly declining slope and a constant threshold level averaging 0.6 m. amp. 3. After 24 hours altered curves of two types appeared (a) one that showed a normal refractory period but a high diastolic threshold level. The elevated threshold was believed due to separation of the electrodes from the myocardium by fibrin deposit. (b) A curve with an abnormal refractory period as well as an elevated diastolic threshold. These latter were found to be associated with pathological changes in the myocardium under the electrodes as well as with the presence of epicardial fibrinosis.

1. Suckling, E. E., Brooks, C. McC., Orias, O., Gilbert, J. L., and Siebens, A. A., *Am. J. Physiol.*, 1950, v162, 213.

2. Brooks, C. McC., Orias, O., Gilbert, J. L., Siebens, A. A., Hoffman, B. F., and Suckling, E. E., *ibid.*, 1950, v163, 469.

3. Sikand, R. S., Nahum, L. H., Levine, H., and Geller, H., *Yale J. Biol. and Med.*, 1952, v24, 366.

4. Delgado, J. M. R., and Sikand, R. S., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 80.

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24-Hour Periodicity and Audiogenic Convulsions in I Mice of Various Ages.*† (21526)

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The work to be reported herein was begun to explore the functional pattern of the daily cycle(1) in I mice, in relation to the problem of the genesis of breast cancer: the 24-hour rhythmical changes in number of circulating eosinophils and in rectal temperature were in-

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vestigated and will be described. But in the course of this work convulsive seizures were occasionally noted while the mice were prepared for venisection (by transfer into a glass jar, at roughly 9 inches under an unglazed 100 watt lamp)(2). To follow up these incidental observations, the effects of auditory stimulation were also studied during opposite phases of the 24-hour cycle. Under these conditions, the I mice were found to be susceptible to audiogenic convulsions and to maintain this susceptibility to seizures over a considerable portion of their life span. Moreover, they exhibited a marked 24-hour periodicity in their susceptibility to audiogenic seizures and, also, in their ability to recover from them. While the investigations were initiated solely to explore some hormonal mechanisms which may contribute to breast cancer(3), this report will also describe the susceptibility to induced convulsions as a characteristic of the I stock of mice and, thus, perhaps, the availability of a tool for work on the mechanisms of periodicity in the occurrence of clinical epileptic seizures(1).

Materials and methods. Mice of the I stock, of both sexes and of various ages, maintained in the Division of Experimental Surgery at the University of Minnesota were used. For 7 days prior to the initiation of the studies, the mice were kept singly housed in a room illuminated by artificial light only. A clock-controlled switch turned the lights on at 06:00 and off at 18:00. Purina Fox Chow and tap water were available to the mice from the time of weaning and throughout the experiments. The several experiments were carried out in succession, at 7-day intervals. In the first experiments, eosinophil counts were made; next, rectal temperatures were measured, and last, the effects of exposure to sound were studied. For one experiment on the temperature rhythm serially dependent sampling was employed, *i.e.,* repeated measurements were made on the same mice in the course of the same experiment. Serially independent sampling was used for the other experiment on the temperature rhythm and for the experiments involving eosinophil counts and auditory stimulation. In this type of sampling, different mice were used at the two times of day chosen for the study. In view of earlier results(1), all of the sampling with a serially independent design was carried out during the periods from 08:00 to 11:00 (day) and from 21:00 to 24:00 (night).

The *procedures* employed for direct eosinophil counts in tail blood(2) and for the measurements of temperature with a thermistor-bridge circuit(4,5) have been described. The method described by Hall(6) as used by Jardetzky(7) was used for auditory stimulation (roughly 104 decibels above 0.0002 dynes/sq cm rms pressure). A "bell-tub" was set up outside of the sound-deadened animal room. The transfer of a mouse in its cage from the animal room to the testing place, including the subsequent transfer of the mouse from cage to tub, was completed within one minute. A switch was then thrown to ring the bells, and the stimulation was continued for a one-minute period, measured by means of a stop-watch. The mice were tested in succession, one at a time, during the 2 periods of the day indicated above. A convulsion was recorded if the mouse fell on its side and exhibited rapid spasmodic movements of its legs ("clonic"), or if it turned over on its back with all 4 legs brought close to the body and then gradually extended the hind legs straight backward ("tonic"). Wild running and dashing consistently preceded the convulsions; this agrees with earlier observations (8,9).

Results. The variability encountered in the eosinophil counts of the mice studied may be seen in Fig. 1. This considerable variability notwithstanding, day-night differences in mean counts may be discerned from this figure. These day-night differences are statistically significant for the several age-groups studied ($P<.05$). Day-night differences also were encountered in the mean rectal temperatures of these mice and the differences were significant below the 1% level. Consistently, the mean rectal temperatures were high by night and low by day, irrespective of the use of serially dependent or independent sampling procedures. An analysis of variance carried out on the data obtained with serially independent sampling further revealed significant age and sex differences in

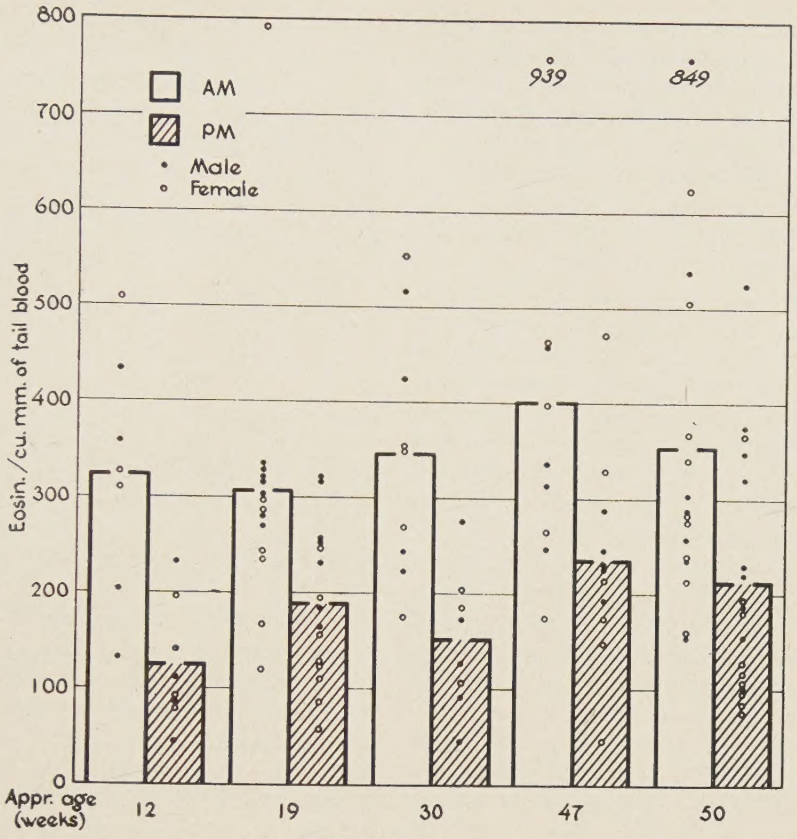

FIG. 1. Eosinophil rhythm in groups of I mice of various ages. Serially independent sampling.

rectal temperature. On the average, the mean rectal temperatures showed a decreasing trend with age; also, for I mice 12 and 19 weeks of age, the mean rectal temperatures were significantly lower in females than in males.

Fig. 2 shows the convulsive response exhibited by the several groups of mice during auditory stimulation by the bell-tub method. It is readily apparent from this figure that the effects of stimulation at the two different times of day were not the same. The incidence of convulsions was high during stimulation by night but low during stimulation by day. Moreover, the ability of the mice to recover from convulsions was low by night but high by day. The periodicities in the convulsion risk and in the mortality from convulsions are statistically significant ($P<.01$). A decrease in the susceptibility to audiogenic convulsions with advancing age may also be seen in Fig. 2. Similar decreases in susceptibility with age have been noted for other

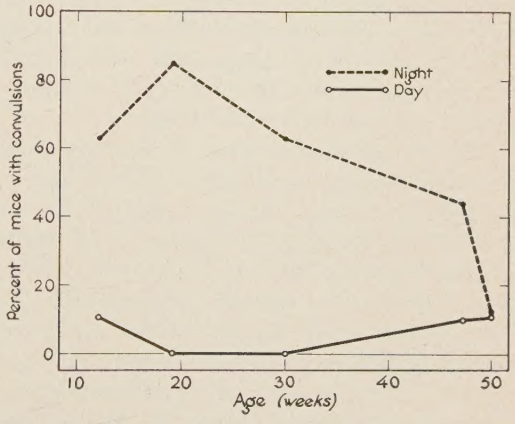

FIG. 2. Periodicity in convulsions of *"I" mice* of various ages, exposed to "bell-tub." Serially independent sampling. 62 mice were tested by day, another 55 mice at night: 4 convulsions (1 fatal) were noted by day, 27 convulsions (all fatal) were noted at night.

stocks(10-12), with the interesting exception of the extreme dilution stock(13), in which the susceptibility increases with age. In view of the sample size employed, we must refrain, however, from comments concerning the question whether the age-change in over-all susceptibility to audiogenic convulsions is also associated with changes in the periodicities described herein for convulsions.

Discussion. The findings of day-night differences in eosinophil count and in rectal temperature in I mice are in agreement with the results of earlier work(14-18). Moreover, the data on rectal temperatures, if pooled irrespective of the time of day when they were obtained, reveal a decrease in temperature with age, and also significant sex differences for the younger age groups. Further sex and age differences, namely, differences in the amplitude of the daily cycles in mean temperatures, are apparent from the periodograms (19) presented in Fig. 6. It is pertinent to note in this connection that a decrease in the amplitude of the mean curves is not necessarily indicative of a decrease in the amplitude of the curves for individual mice. This decrease in amplitude of mean curve may be brought about, in part, by a lesser degree of synchronization of the individual curves. A slight lengthening of the period of the cycle, perhaps a result of handling the mice for measurement, may also be seen for nine of the ten periodograms shown in Fig. 3. But definition of changes in amplitude and period of cycle and of the physiologic mechanisms involved must await the analysis of more extensive data on individual I mice.

The 24-hour periodicities in susceptibility to sound-induced convulsions and in ability to recover from convulsions are the main point of this report. The significance of these phenomena may have been obscured in the past, more by the lack of standardization of the physical environmental factors which so markedly influence 24-hour periodicity than by the use of serially dependent sampling. Nevertheless, similar trends, at least in the susceptibility to auditory stimulation, may be seen in data obtained by earlier investigators (7,20). With the experimental design employed herein, the over-all susceptibility to

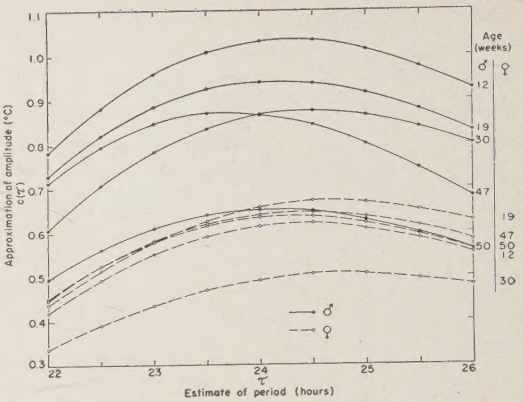

FIG. 3. Periodograms of mean rectal temperatures obtained (at 4-hr intervals over a 4-day period) on groups of I mice of both sexes and various ages. Note, 1) smaller amplitude of mean curves for females (except for 50 wk age groups); 2) decrease in amplitude with age, for male groups; 3) period of cycle is slightly longer than 24 hr in 9 of 10 periodograms shown (see text).

audiogenic seizures (data pooled irrespective of age) was 6% by day and 49% by night. Moreover, of the 27 mice convulsing by night, none recovered from seizure, while 3 of the 4 mice convulsing by day did recover. Thus, comparable mice, tested by the same auditory stimulus, may be described either as highly susceptible or as non-susceptible to convulsions, depending upon the time of day chosen for stimulation. Unawareness of the occurrences of daily physiologic changes in convulsive response from 11% by day to 63% by night in one group, or from 0% by day to 85% by night in another group (Fig. 2) is likely to constitute a considerable source of variation in some experimental data. But the phenomenon of convulsive periodicity is of interest in itself. Its study may yield new clues for the analysis of the sequence of events underlying the activity cycle(1). Thus, clues may also be obtained concerning the mechanisms which contribute to the unequal distribution of seizures during the hours of the day in certain epileptics, a subject which has recently been reviewed(1).

Summary. Daily rhythms were studied in I mice of several age groups under standardized experimental circumstances. The rhythms in eosinophil count and in rectal temperature are described for this stock. Moreover, 24-hour periodicities are noted for

the susceptibility of I mice to audiogenic convulsions and for their ability to recover from convulsions. The magnitude of these differences was large and was statistically significant. In one group of young animals the convulsive risk was 11% by day and 63% by night and in another, 0% by day and 85% by night. Changes with age in several of the variables studied are also noted.

1. Halberg, F., *J. Lancet* (U. S. A.), 1953, v73, 20.
2. Halberg, F., Bittner, J. J., and Visscher, M. B., *Blood,* 1951, v6, 832.
3. Bittner, J. J., *Cancer Res.,* 1952, v12, 594.
4. Becker, J. A., Green, C. B., and Pearson, G. L., *Trans. Am. Inst. E. E.,* 1946, v65, 711.
5. Halberg, F., Zander, H. A., Houglum, M. W., and Mühlemann, H. R., *Am. J. Physiol.,* 1954, v177, 361.
6. Hall, C. S., *J. Heredity,* 1947, v38, 3.
7. Jardetzky, O., Convulsions in C_3H male mice in relation to dietary fat, M. S. Thesis, U. of Minn., 1953.
8. Dice, L. R., *J. Mammal.,* 1935, v16, 25.
9. Antonitis, J. J., *Science,* 1954, v120, 139.
10. Watson, M. L., Contrib. Lab. Vert. Gen. No. 11, U. of Michigan, 1939.
11. Ginsburg, B., and Huth, E., *Genetics,* 1947, v32, 87.
12. Vicari, E. M., *ibid.,* 1948, v33, 128.
13. ———, *ibid.,* 1950, v35, 697.
14. Halberg, F., and Visscher, M. B., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 846.
15. Halberg, F., Levy, L., and Visscher, M. B., *Federation Proc.,* 1953, v12, 59.
16. Halberg, F., Bittner, J. J., and Visscher, M. B., *Am. J. Physiol.,* 1953, v174, 313.
17. Panzenhagen, H., and Speirs, R. S., *Blood,* 1953, v8, 536.
18. Louch, C., Meyer, R. K., and Emlen, J. T., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 668.
19. Whittaker, E. T., and Robinson, G., *The Calculus of Observations,* pp. 343-362, Blackie and Son, London, 1924.
20. Patton, R. A., Karn, H. W., and King, C. J., *J. Comp. Psychol.,* 1942, v33, 253.

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Antigenic Comparisons of Strains of *Pasteurella pestis.* (21527)

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Various experimental methods employed by Schutze(1), Wats *et al.*(2), and Jawetz and Meyer(3,4) directed toward the problem of distinguishing between virulent and avirulent strains by serological methods have failed to detect notable qualitative differences between any strains of this organism. On the other hand, Fadeeva(5), Gheltenkoff and Khvorostukina(6), and Khorobkova(7), believed that such differences were demonstrable.

The maximum number of antigens which *P. pestis* might possess is unknown. Schutze (8) postulated 2, a somatic and an envelope antigen. Baker *et al.*(9) were able to isolate and purify 2 fractions, Fraction IA and IB. A third, Fraction II, was concentrated but not purified. Seal(10) reported the isolation of 5 different fractions from *P. pestis.* Preliminary observations of the authors using the technic of Oakley and Fulthorpe(11) indicated the presence of as many as 10 different precipitation zone-producing components in virulent and avirulent strains of *P. pestis.* Even fractionated components of strains Yreka and 14, as well as samples of Fraction IA, produced 4 zones or more. If *P. pestis* is possessed of so complex an antigenic structure as that indicated by diffusion-precipitin methods, the question arises as to whether this complex pattern is duplicated between different strains, or whether there might be differences giving rise to definable patterns reminiscent of the antigenic patterns of the Salmonellae.

The present paper describes data indicating that certain differences and similarities exist between strains of *P. pestis* which are distin-

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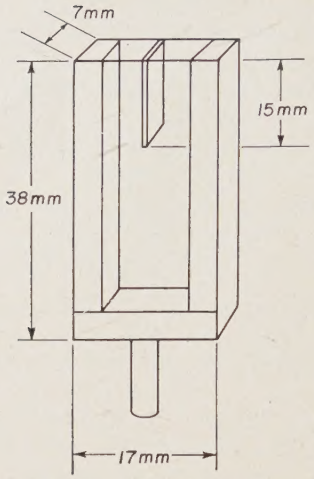

FIG. 1. Modification of Antigen Comparator Cell of Surgalla, *et al.*(12).

guishable by means of an antigen comparison technic.

Methods and materials. After incubation for 4 days at 37°C, growth of *P. pestis* was harvested in 0.85% NaCl solution. The suspensions were stored at –20°C until they were removed and thawed for testing by means of the technic described below. Fig. 1 shows the 2-channel comparator cell which was adopted for this work. It is a modification of the 4-channel cell used by Surgalla *et al.*(12). The latter was given an extensive trial, but patterns produced were often complex and difficult to interpret. Very few such difficulties have been encountered with the 2-channel modification; in addition, the smaller volume effects a considerable economy in the use of antiserum. The component parts were cut from Plexiglass of 1/16 inch thickness and cemented together with methylene dihydrochloride. The peg extending from the base of the cell facilitates handling and racking in ¼-inch holes drilled into Lucite blocks. In use, the cells were pre-coated with 1% agar (13). The cells were filled with antiserum-agar mixture just to the lower end of the center dividing strip. After the serum-agar mixture had solidified, the 2 suspensions to be compared were placed one on each side of the center dividing strip in contact with the agar meniscus. A partially purified globulin concentrate of rabbit antiplague serum† was made up in a stock dilution of one part of reconstituted serum to 9 parts of sterile 0.85% saline buffered to pH 7.0. This preliminary dilution was necessary because direct dilution of the serum in melted agar invariably resulted in a hazy serum-agar mixture. The final mixture was prepared by diluting the stock 1:10 antiserum 1:5 in melted agar clarified by centrifugation(14) and containing 1% NaCl and 1:2000 sodium ethylmercurithiosalicylate (merthiolate). Thus, the final dilution of reconstituted rabbit antiplague serum was 1:50. Dilution of serum in melted agar was carried out after both ingredients had been brought to 50°C in a water bath. After the comparator cells were set up as described, the open ends were sealed with Parafilm and placed in a 30°C incubator. Readings were made at 3- and 7-day intervals both visually and by photographing the cells with a 35-mm single lens reflex camera (Exakta) fitted with an extension tube. The method of lighting was critical. Good results were obtained when light from a No. 1 Photoflood bulb, diffused through ground glass in an X-ray film viewer, was used to illuminate the cells from below and behind. A black paper background was used. The results are shown in Fig. 2-3. Three avirulent and 2 virulent strains of *P. pestis* were selected for comparison with one another: A1122, E.V. 76, and 14 (avirulent); 195/P and Yreka (virulent). All suspensions were handled with bacteriological safety methods(15).

Results. When suspensions of the same strain were placed in both channels of the comparator cell, precipitation zones resulting from diffusion of antigens into the serum-agar mixture extended in even lines all the way across the cell. This is to be expected when all antigens which can be defined by this method are common between the two suspensions, and this picture was always seen when like suspensions were placed on both sides of the comparator cell.

A similar picture was obtained when the following strains were compared with one another: E.V. 76 (avirulent) with Yreka (virulent); E. V. 76 (avirulent) with 195/P (virulent), 195/P (virulent) with Yreka (viru-

† E. R. Squibb & Son.

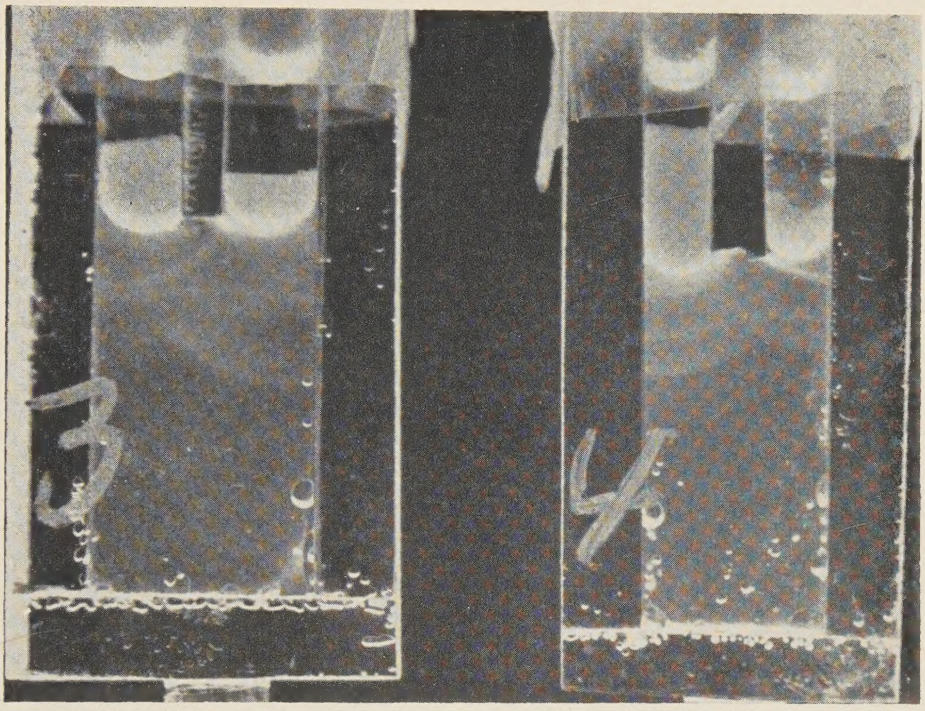

FIG. 2. Comparator cell on left of photograph is a match between *P. pestis* strain A1122 and strain 14; cell on right is a match between A1122 and Yreka. In each case, A1122 suspension was placed in left channel. A single homologous component, extending across both sides of cell. Other antigens are heterologous, developing a semi-radial pattern. Photograph made at 3 days.

lent). Four separate precipitation zones could be visualized in each of the 3 matches.

In all other cases, patterns of crossing zones were produced, indicating non-homology of the strains compared. Thus, in the A1122-14 match (Fig. 2), 4 zones could be seen arising from each side of the comparator cell. Only one of these is common to both organisms, a zone of intermediate mobility extending all the way across the cell. It will be noted that the components of least mobility cannot be strictly compared, being too close to the meniscus to permit contact. The common zone between these two organisms was identifiable also in all other matches. Partial heterogeneity is also indicated in the A1122-Yreka match on the right of Fig. 2.

Discussion. According to current interpretations(13) of diffusion-precipitation systems of the type described, merging lines arising as a consequence of diffusion of antigens in a common direction from separate sources are considered to indicate the presence of identical, or at least very closely related, antigens. In the 2-channel comparator cell, quantitative as well as qualitative similarity would be indicated when the precipitation zones extend in an even line all the way across the cell. Where there is appreciable dissimilarity in concentration of an antigen, a radial pattern should be produced arising from the side characterized by the higher concentration, which would merge with a brief transverse line arising from the side of lower concentration. Such a pattern is visible in Fig. 3. When an antigen present on one side is completely lacking on the other, a complete semi-radial pattern should be apparent, and this is found in the case of many components in matches between strain A1122 and all other strains tested. By means of this type of analysis, it is possible to divide the 5 strains into 3 groups indicating antigenic relationships: Group I, comprising strains 195/P, Yreka, and E. V. 76; Group II, comprising strain A1122; Group III, comprising strain 14. The last of these, strain 14, also has close relationships with strain Yreka (Fig. 3).

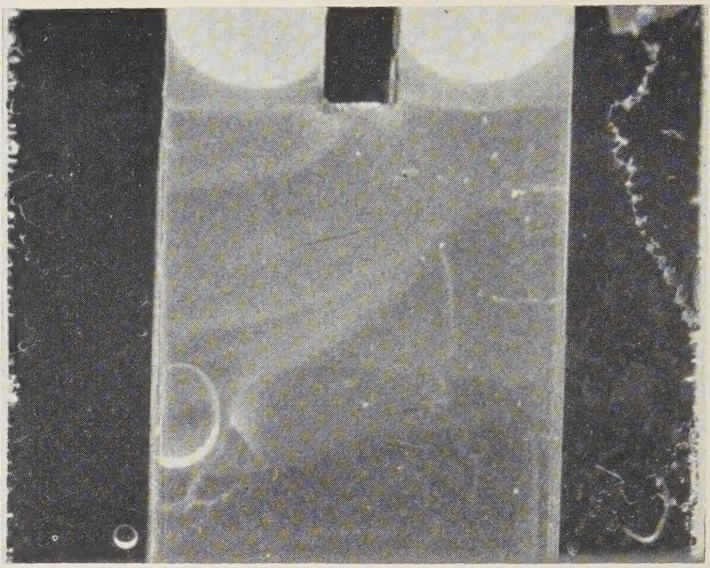

FIG. 3. A match between *P. pestis* strain Yreka (left) and strain 14 (right). Pattern is a mixture of homologous and heterologous zones. Acetone-killed and dried organisms used. Photograph taken at 10 days. Small bubble has appeared in serum-agar at this time period.

The individuality of strain A1122 is particularly surprising because this strain was the one used to produce the antiserum. There is no experimental evidence to explain this result as yet. Our working hypothesis is as follows: All antigenic components of strains other than strain A1122, perhaps with the exception of the one common antigen seen in all paired comparisons, must be cross-reacting (16) antigens, which are sufficiently different from the homologous A1122 antigen to possess different diffusion coefficients.

A hypothesis which seems less likely to us is that differences in relative concentration of homologous antigens on opposite side of the comparator cell might produce patterns interpretable as due to non-homologous antigens. Thus, the boundary of a highly concentrated antigen on one side might lead its homologue on the opposite side so much as to avoid merging with it.

Summary. 1. Diffusion of mixed antigens from different sources in parallel directions in serum-agar gels was used for comparing the antigenic structure of 3 avirulent and 2 virulent strains of *P. pestis*.

2. Merging of precipitation zones is a criterion of homology of antigens; crossing of zones is a criterion of heterology. By these criteria 3 groups among 5 strains of *P. pestis* were established: Group I, Strains 195/P, Yreka, E. V. 76; Group II, Strain A1122; Group III, Strain 14.

1. Schutze, H., *Brit. J. Exp. Path.,* 1939, v20, 235.

2. Wats, R. C., Wagle, P. M., and Puduval, T. K., *Indian J. Med. Res.,* 1939, v27, 373.

3. Jawetz, E., and Meyer, K. F., *J. Inf. Dis.,* 1943, v73, 124.

4. ———, *Am. J. Path.,* 1944, v20, 457.

5. Fadeeva, T., *Rev. Microbiol. Saratov,* 1939, v18, 44.

6. Gheltenkoff, A., and Khvorostukina, M., *ibid.,* 1940, v19, 194.

7. Korobkova, E., *Ibid.,* 1937, v16, 1. Abstracted in: *Bull. Off. Internat. d'hyg. pub.,* 1939, v31, 505.

8. Schutze, H., *Brit. J. Exp. Path.,* 1932, v13, 284.

9. Baker, E. E., *et al., J. Immunol.,* 1952, v68, 131.

10. Seal, S. C., *Ibid.,* 1951, v67, 97.

11. Oakley, C. L., and Fulthorpe, A. J., *J. Path. Bact.,* 1953, v65, 49.

12. Surgalla, M. J., *et al., J. Immunol.,* 1952, v69, 357.

13. Oudin, J., *Methods in Med. Res.,* 1952, v5, 335.

14. Munoz, J., and Becker, E., *J. Immunol.,* 1950, v65, 47.

15. Wedum, A. G., *Am. J. Pub. Health,* 1953, v43, 1428.

16. Kabat, E. A., and Mayer, M. M., *Experimental Immuno-chemistry,* 1st Ed., 1948, Chas. C. Thomas, Springfield, Ill., p216.

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Diet of Adult Female Rats and Relation to Kidney Lesions in Choline Deficient Offspring.* (21528)

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It has been reported that an 18% casein diet with added cystine and low in choline produces renal lesions in 95% of young male rats in an 8-day period(1,2). We have had difficulty in duplicating these results in our animals (Sprague-Dawley) and have undertaken a study on the influence of the mother's diet during the suckling period on the incidence of kidney lesions in the offspring. The present communication gives results which indicate that vit. B_{12} added to the mother's diet during the period from birth to weaning of her young lowers the incidence of renal lesions in the young consuming a low choline diet.

Experimental. Adult female rats of the Sprague-Dawley strain having received Purina Laboratory Chow since birth were placed in raised cages and fed experimental diets *ad libitum* for various lengths of time before weaning of their young. When the litters reached 21 to 22 days of age they were placed in raised cages and fed a low choline diet *ad libitum*. At the end of a 7 day experimental period the young were sacrificed by decapitation and the kidneys examined for hemorrhages. The appearance of the kidneys is recorded in Table I as normal or hemorrhagic. The latter designation includes those that were slightly hemorrhagic, moderately hemorrhagic and severely hemorrhagic. The mothers were divided into 4 groups and fed the diets shown in Table I for various lengths of time before weaning of their young. Diet TC2 is the basal low choline diet consisting of casein (General Biochemicals, Inc.) 18%, dry brewers yeast (Nutritional Biochemicals) 6, agar 2, salt mixture (General Biochemicals Inc. #XIV) 4, cane sugar 48.7, lard 19.9, Natola 0.1, calcium carbonate 1.0 and l-cystine 0.3. The crystalline vit. B_{12} used in these experiments was obtained from Nutritional Biochemicals.

TABLE I. Effect of Vit. B_{12} and Choline Content of Mother's Diet on Incidence of Renal Lesions in Young Rats on a Low Choline Diet (TC2) for 7 Days.

Diet group and time mothers on diet*	No. and sex of young with normal kidneys		No. and sex of young with hemorrhagic kidneys	
1 (21)*	1 ♂	2 ♀	9 ♂	12 ♀
(14)	3 ♂	6 ♀	8 ♂	7 ♀
(7)	3 ♂	5 ♀	9 ♂	2 ♀
2 (21)	5 ♂	13 ♀	9 ♂	0 ♀
(14)	6 ♂	11 ♀	8 ♂	0 ♀
(7)	0 ♂	3 ♀	12 ♂	6 ♀
3 (21)	9 ♂	8 ♀	5 ♂	0 ♀
(14)	7 ♂	16 ♀	0 ♂	0 ♀
(7)	5 ♂	10 ♀	3 ♂	0 ♀
4 (21)	10 ♂	9 ♀	1 ♂	1 ♀
(14)	7 ♂	10 ♀	3 ♂	0 ♀
(7)	5 ♂	10 ♀	0 ♂	3 ♀

* No. in parenthesis indicates days mothers on diet before weaning young.

Diet 1—TC2.
2— " + 1 mg choline chloride/g food.
3— " + 0.06 γ vit. B_{12}/g food.
4— " + 1 mg choline chloride + 0.06 γ vit. B_{12}/g food.

Results. The effects of the various supplements in the mother's diet on the incidence of renal lesions in her young after 7 days on diet TC2 are also shown in Table I. Mothers in group I were fed basal diet TC2 with no added supplement. Out of a total of 33 males born in this group 26 had hemorrhagic kidneys after 7 days on diet TC2. Of a total of 34 females, 21 had hemorrhagic kidneys. Mothers in group 2 were fed diet TC2 supplemented with 1 mg added choline chloride per gram of food. Of a total of 40 males born in this group, 29 had kidney lesions after 7 days on diet TC2, while out of 33 females, 6 had lesions. Mothers in group 3 received diet TC2 supplemented with 0.06 γ of crystalline vit. B_{12} per gram of food. Out of a total of 29 males and 34 females born in this group,

* This investigation was supported by a research grant from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, Public Health Service.

8 males and no females had hemorrhagic kidneys after 7 days on diet TC2. In group 4 the mothers received diet TC2 supplemented both with 1 mg choline chloride and 0.06 γ vit. B_{12} per gram of food. Of a total of 26 males and 33 females born in this group, 4 males and 1 female had renal lesions after 7 days on diet TC2.

The data indicate that 0.06 γ added vit. B_{12}/g of food in the mother's diet prior to weaning reduced the incidence of hemorrhagic kidneys in her young consuming a choline low diet. Choline chloride at a level of 1 mg/g of food had little effect on the incidence in the males but a more pronounced effect in the females (Group 2).

In another experiment one group of adult females was fed Purina Laboratory Chow plus a daily supplement of 2 to 3 g of canned dog food (Strongheart) for 42 days before weaning of the young. Another group was fed Rockland Complete Rat Diet for 42 days before weaning. The young males from each litter were placed on the basal diet TC2 for 6, 7 and 9 days. At the end of these periods they were sacrificed and the kidneys examined for hemorrhages. Of a total of 52 males born to mothers on Purina Laboratory Chow plus canned dog food only one had hemorrhagic kidneys. Of a total of 29 males born to mothers on Rockland Complete Rat Diet, 23 had hemorrhagic kidneys. Ralston Purina Co. adds vit. B_{12} feed supplement to the Laboratory Chow. Rockland Farms do not add vit. B_{12} to their Rat Diet (Complete) (3).

Discussion. It is not surprising that the vit. B_{12} content of the mother's diet affects the incidence of hemorrhagic kidneys in young rats on low choline diets. It has been shown (4,5,6,7) that rats are able to store vit. B_{12} and that the mother during the suckling period is able to transmit vit. B_{12} to the young in her milk. Others have observed (8,9,10) a marked relationship of vit. B_{12} to the incidence of hemorrhagic kidneys in young rats. Our results suggest that during the suckling period if the mother's diet contains a good level of vit. B_{12} the baby obtains enough B_{12} from the milk to protect it against kidney lesions during the critical period of 6 to 10 days after weaning. The possibility that the young animals may eat enough of the mother's diet a few days before weaning to protect them cannot be ruled out. Further study should answer this question.

Summary. The incidence of hemorrhagic kidneys in young rats after seven days on a diet containing 18% casein, 0.3% added cystine and low in choline is markedly reduced if 0.06 gamma vit. B_{12}/g of food is incorporated in the diet of the mother during the period from birth to weaning. Choline when added at 1 mg/g of food affords very little protection in young males when compared to that shown by 0.06 gamma of vit. B_{12}. This level of added choline (1 mg/g of food) has a greater protective effect in the females.

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1. Griffith, W. H., *J. Nutrition,* 1941, v21, 291.

2. Griffith, W. H., and Mulford, D. J., *J. Am. Chem. Soc.,* 1941, v63, 929.

3. Personal communication.

4. Daniel, L. J., Gardiner, M., and Ottey, L. J., *J. Nutrition,* 1953, v50, 275.

5. Dryden, L. P., Hartman, A. M., and Cary, C. A., *ibid.,* 1952, v48, 509.

6. Lewis, U. J., Register, U. D., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 509.

7. Richardson, L. R., Witten, P. W., and Couch, J. R., *ibid.,* 1951, v76, 265.

8. Schaeffer, A. E., Salmon, W. D., Strength, D. R., and Copeland, D. H., *J. Nutrition,* 1950, v40, 95.

9. Strength, D. R., Schaeffer, A. E., and Salmon, W. D., *ibid.,* 1951, v45, 329.

10. Hawk, E. A., and Elvehjem, C. A., *ibid.,* 1953, v49, 495.

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Effect of B-Complex Deficiencies on Rat Serum Complement.* (21529)

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The impairment of antibody response in certain specific vitamin deficiencies in the rat has been demonstrated(1). Serum complement is another important component of many immune systems. In addition, it is a biologically-active and hence readily measured protein complex which is normally present in the blood. It does not require any special stimulus for its synthesis analogous to that of antigen for antibody production. An investigation of serum complement in vitamin-deficient rats should, therefore, be of value in determining the relationship of antibody synthesis to the synthesis of other serum proteins. Earlier work on the effect of vitamin deficiencies on the level of serum complement yielded contradictory results(1). Wertman *et al.*(2) have recently observed a virtually complete loss of complement activity in niacin-tryptophane deficiency of the rat. Complement activity of the inanition controls was also severely depressed. Complement values obtained by the present authors in preliminary experiments in several vitamin deficiency states have been reported(3).

In the present paper serum complement activities of biotin, pteroylglutamic acid (PGA), riboflavin and pyridoxine-deficient rats will be presented. The parenteral injection of an antigen initiates a process resulting in the appearance of a new serum protein. It might therefore be of interest to study the regeneration of serum complement following its *in vivo* depletion in order to compare it with the production of antibody. In addition, the ability to regenerate complement can be assumed to represent a severe test of the adequacy of the mechanisms involved in complement synthesis. Accordingly, the regeneration of serum complement following its *in vivo* depletion in riboflavin and pyridoxine-deficient and control rats was determined and will be reported in this paper.

Methods. Animals and Diets. Male weanling albino rats of the Sprague-Dawley strain were employed. The animals were housed individually in wide-meshed, screen bottom cages. The pyridoxine and riboflavin-deficient animals were fed the basal diet of the following percentage composition: Sucrose, 56.76; "vitamin-free" casein, 25.00; salts(4), 4.00; cod liver oil, 2.00; hydrogenated vegetable oil, 10.00; corn oil, 2.00; choline chloride, 0.20; *i*-inositol, 0.03; *dl*-α-tocopherol acetate, 0.01; and 2-methyl-1, 4-naphthoquinone, 0.001. The PGA-deficient animals were fed the same basal diet with the exception that 1.0% sulfasuxidine was added at the expense of sucrose. The biotin-deficient animals were fed the basal diet in which 60% of the total casein used was replaced by dried egg white. A control group for each of the deficiencies received the same basal diet as the corresponding deficient group. All animals were fed *ad libitum* with the exception of a group of inanition controls which was paired-fed with the riboflavin-deficient animals. In addition each animal received a vitamin pill daily. Each of the pills fed the control groups supplied the following vitamins: thiamin, 40 γ; niacin, 100 γ; riboflavin, 60 γ; calcium pantothenate, 200 γ; pyridoxin, 50 γ; biotin, 1 γ; PGA, 1 γ, with the exception that the biotin control pill contained 4 γ of biotin. For each of the deficient groups the appropriate vitamin was omitted from the pill. *Complement Assay.* Animals were placed under nembutal anaesthesia and blood drawn by cardiac puncture. The serum was separated and assayed immediately or quick-frozen and stored at –30°C for a period not exceeding 2 weeks. Complement was assayed using 1 ml of 1.25% sheep cells sensitized with 4 units of amboceptor in a total volume of 1.5 ml. Incubation was at

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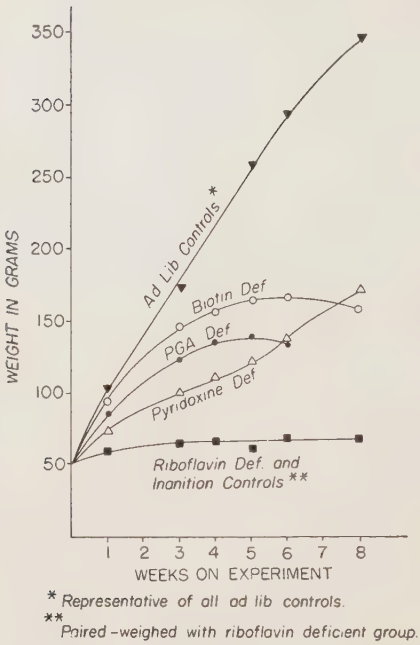


FIG. 1. Growth curves of vitamin-deficient rats.

37°C for ½ hour and the endpoint estimated by visual comparison with standards representing 0-100% lysis in 10% steps. Titers are expressed in 50% units per ml of serum. One such unit is the amount of complement necessary to lyse 50% of the cells present under the conditions of assay. *In Vivo Depletion of Serum Complement.* Depletion was accomplished by intraperitoneal injection of 0.1 mg. Type III pneumococcal polysaccharide‡ followed 1 hour later by injection into a tail vein of a neutralizing amount of homologous rabbit antiserum.‡ The general procedure followed was that of Seltzer *et al.* (5). Three successive daily depletions were employed. At least 80% of the hemolytic activity was destroyed 1-3 hours after the final injection. Samples for the regeneration study were drawn 22 hours after the last injection. Previous experiments indicated that 22 hours was the optimal time to compare the rates of regeneration in the groups investigated.

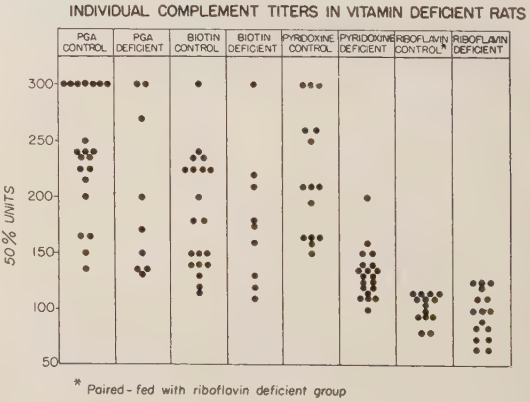
Results. Growth responses of the various groups are shown in Fig. 1. Serum complement was determined after 8 weeks on experiment with the exception of the PGA-deficient group and its controls where the assays were performed after 6 weeks. Serum complement titers of the individual rats are given in Table I. Wide variations within each group are apparent. A reduction in complement activity is noted, however, in the riboflavin-deficient animals and their inanition controls and in the pyridoxine-deficient animals.

One week after the determination of the complement titer, serum complement was depleted *in vivo* and the extent of complement regeneration determined as described above. The results are given in *Table II.* The rates of serum complement regeneration were essentially the same in all groups. Similar results were obtained in preliminary studies on biotin and PGA-deficient rats and in inanition controls.

Discussion. The deleterious effect of a decreased food intake *per se* upon the serum complement level has been clearly demonstrated in these experiments. The lowered complement titers of the riboflavin and pyridoxine-deficient rats can be attributed to the state of inanition in these deficiencies. Comparison of the growth curves (Fig. 1) and serum complement titers (Table I) reveals a parallelism between final complement titer and growth during the major part of the experiment. The acute depression of growth during the last 2-3 weeks in the biotin and PGA-deficient groups did not affect the complement level. It is probable that many of the controversial results obtained in studies of the relationship of nutritional factors to

TABLE I

INDIVIDUAL COMPLEMENT TITERS IN VITAMIN DEFICIENT RATS

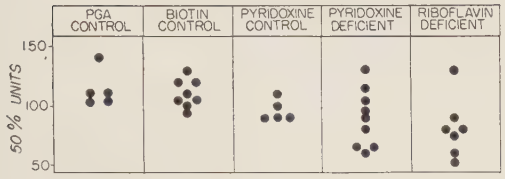


* Paired-fed with riboflavin deficient group

‡ Kindly supplied by Dr. J. O. Lampen, Squibb Institute for Medical Research.

TABLE II

SERUM COMPLEMENT REGENERATION IN INDIVIDUAL VITAMIN DEFICIENT RATS.*



*Complement Levels Attained 22 Hours After Third Successive Daily Depletion.

serum complement activity can be attributed to the failure to recognize this effect of inanition.

The serum complement values 22 hours after depletion are the same in both the control and deficient rats (Table II). In the controls, complement continues to be regenerated until the pre-depletion steady-state values shown in Table I are attained within 48 hours following depletion. In contrast, little further regeneration occurs in the pyridoxine and riboflavin-deficient groups. In these two groups the complement titers also return to their original steady-state values which are approximately one-half of the control values.

These studies were undertaken to gain an insight into the process of antibody synthesis and how it is affected in vitamin deficiencies. The results with complement reported in the present paper and previous studies on the levels and distribution of serum proteins in vitamin deficiency states(3) have failed to show any specific effect upon serum protein or complement. In contrast, specific effects of certain deficiencies on antibody formation have been demonstrated. It would, therefore, seem that the mechanism for the biosynthesis of antibody differs from that of serum protein and complement.

Summary. 1. Serum complement titers were studied in pteroylglutamic acid, biotin, riboflavin and pyridoxine-deficient rats and their respective controls. A decreased level of complement was noted only in the pyridoxine and riboflavin-deficient rats. This effect could be attributed to the concomitant state of inanition in these deficiencies. 2. The rates of serum complement regeneration in pyridoxine and riboflavin-deficient rats were similar to those of control animals.

1. Axelrod, A. E., and Pruzansky, J., *Vitamins and Hormones,* in press.

2. Wertman, K., Smith, L. W., and O'Leary, W. M., *J. Immunol.,* 1954, v72, 196.

3. Axelrod, A. E., and Pruzansky, J., *Symposium on Protein Metabolism, Nutrition Symposium Series,* No. 8, p26, 1952, The National Vitamin Foundation, New York, N. Y.

4. Jones, J. H., and Foster, C. J., *J. Nutrition,* 1942, v24, 245.

5. Seltzer, G., Baron, S., and Fusco, J., *J. Immunol.,* 1952, v69, 367.

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Neutralization and Precipitation of the Virus of Avian Erythromyeloblastosis with Serum of Hyperimmunized Chickens.*† (21530)

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A serious impediment to the study of the virus of erythromyeloblastosis(1,2), as well as the agent or agents of other forms of the avian leukosis complex(3,4), has been the

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lack of specific immune serums. Antiserums neutralizing and fixing complement with the agents of representative types of the complex, sarcoma 13 and erythroleukosis(5-7), for example, have been produced in rabbits with concentrates of the respective viruses. Such antiserums, however, fix complement also in the presence of homogenates of normal chick tissue and, consequently, the results of immunological reactions with them cannot be employed as criteria specific to the properties of the virus. It has been reported occasionally(8,9) that neutralizing antibodies may occur in chickens with erythromyeloblastosis but no means has been described for production of such antibodies by immunization procedures. In recent work in this laboratory (10-11), the virus particles of one strain of erythromyeloblastosis have been concentrated and purified by the ultracentrifugal fractionation of plasma from chicks with this disease. Injection of chickens with such concentrates treated with formalin augmented by repeated administration of highly infectious plasma results in the regular formation of antiviral immune bodies. These antibodies neutralize virus infectivity and precipitate the agent and the specific enzymatic activity to dephosphorylate adenosine triphosphate(12-14) associated with the virus. The results of studies with these antiserums are described in the present paper.

Materials and methods. The virus was the B.A.I. Strain A of erythromyeloblastic leukosis described(1,2) in earlier reports. The agent is a particulate material(10) occurring in high concentration(12), of the order of 10^{12} particles per ml, in the plasmas of some chicks with the disease. Only those plasmas containing particles of this order of numbers were used either directly in the neutralization and precipitation studies or to provide the virus concentrates for precipitation tests. Donors of such plasmas were selected by preliminary estimation of enzyme activity to dephosphorylate adenosine triphosphate by means of the micro screening procedure(15, 16). Blood was drawn from the heart into heparin, freed of cells by centrifugation, filtered with celite and passed through Selas candles in the usual way(1,12). The plasmas were used within a few hours. The concentrated virus for some of the experiments was obtained by ultracentrifugation as previously described(10). For the production of *antiviral immune serums* in chickens, the virus was sedimented a single time and resuspended in Ringer solution in preparations concentrated 10-fold with respect to the initial volume of plasma. It was calculated, on the basis of particle count and density(11), that the plasmas contained about 1.5 mg of hydrated virus per ml and the purified preparations about 15 mg per ml of the agent in the same state. White Leghorn chickens of inbred line 15(17,18), about 3 months old, were injected intravenously 3 times within a week, each with 1.0 ml of concentrate which had been diluted 5-fold with Ringer solution and treated for 20 days at 3°C with formalin in 0.1% total concentration. This dose corresponded to approximately 3 mg of hydrated virus. After an interval of 3 weeks, each chicken was given 1 ml of untreated plasma of high virus content. Two months later, when the chickens had shown no evidence of the disease, two further injections, with an interval of 3 days, were made each with 1.0 ml of plasma. Blood was collected from the heart 10 to 16 days after the last injection. The resulting serum, freed of residual cells, was stored at –16°C and inactivated at 56°C for 30 minutes immediately before test.

The *neutralization experiments* were made with constant volumes of plasma mixed with serial 2-fold dilutions of immune serum. To a volume of plasma, 0.5 to 2 ml, depending on virus content as estimated by ATPase activity or particle count, there was added the appropriate amount of immune serum diluted with BSA-Simms's solution(1) to bring the final volume to 5 ml. Each set of mixtures, together with a normal chicken serum control, was incubated at 37°C for 2 hours. The mixtures were then cooled and kept in an ice bath until they were injected intravenously into 3-day-old White Leghorn chicks of line 15 for measurement of infectivity(19). Potency ratios between the activity of the control plasma preparations and that of the test mixtures with immune serum were determined with the latent period procedure(19). *Pre-*

TABLE I. Neutralization of Virus of Erythromyeloblastic Leukosis with Serum from a Chicken Hyperimmunized with the Virus.

Serum	Virus plasma*	No. particles†	Immune serum*	Chicks inoculated‡	Chicks positive	Median log latent period	% virus neutralized§
	λ	× 10^9	λ			days	
None	7	4.4	—	27	18	1.155	—
	1.4	0.88	—	31	13	1.212	—
Normal	7	4.4	4.0	27	19	1.145	0
Immune	7	4.4	4.0	24	0	—	>99
	7	"	2.0	30	0	—	>99
	7	"	1.0	29	0	—	>99
	7	"	0.5	29	1	>1.3	>99
	7	"	0.25	26	5	1.266	92
	7	"	0.12	24	14	1.220	78

* Vol/0.1 ml inoculum. † No. of virus particles/inoculum. ‡ Corrected No. of chicks inoculated (see (1) for method of calculation). § Calculated on basis of latent period relations(19).

cipitation of virus with the chicken immune serum was observed as macroscopically visible flocculation. For these studies purified concentrates like those described above were employed in 0.1-ml volumes to which there were added 0.2 ml of 15% NaCl solution(20) and 0.1 ml of serial 2-fold dilutions of the immune serum. These mixtures, in capped tubes, were incubated at 37°C for 2 hours and then kept overnight at 3°C. The character of the precipitin reaction occurring in mixtures of immune serum with plasma containing virus was followed also by electron microscopy. In this type study 0.1 ml of whole inactivated immune serum was diluted with 0.1 ml of 15% NaCl solution and added to 0.1 ml of virus-containing plasma dialyzed overnight at 3°C against 8% NaCl solution. The mixture was incubated at 37°C and, at suitable intervals, was sampled in 10 λ volumes which were diluted, immediately, 200-fold with physiological saline solution. An aliquot of 1 ml was spun at 16,300 x g for 30 minutes to sediment the virus onto an agar surface(21). The virus material was fixed with osmic acid vapor while on the agar, stripped off the agar with a collodion film, and shadowed for electron microscopy.

The ATPase activity of the virus preparations and of significant reaction mixtures was estimated by the determination of phosphorus with the method of Fiske and Subbarow(22). Enumeration of virus particles was made by the procedures(21) of sedimentation on an agar surface and counts of the particles in the electron microscope.

Results. Neutralization: The findings in a typical neutralization test are summarized in Table I. It was evident from the last column that the immune serum was strongly neutralizing. This was clearly indicated by the results of quantal response in terms of the incidence of positive inoculations, but accurate calculations of % virus neutralized could be made only on the basis of the latent period procedures(19). Though no response was obtained with the largest volumes of immune serum, the actual amount of virus neutralized could not be determined. The median effective dose of virus in this experiment was 1.44 x 10^9 virus particles, and amounts of virus insufficient to initiate infection in these relatively highly resistant hosts may have escaped the action of the immune serum. Nevertheless, with the smallest volume of immune serum, the mixture behaved as if only

TABLE II. Precipitation of Virus of Erythromyeloblastic Leukosis with Serum of a Chicken Hyperimmunized with the Virus.

	Dilution of antigen				
	Undil.	1:2	1:4	1:8	1:16
Dilution of antibody	No. of particles × 10^{10}				
	17.1	8.6	4.3	2.1	1.1
Undil.	++*	++	+	±	0
1:5	++	++	+	0	0
1:20	0	0	0	0	0

* ++ = complete precipitation; + = < complete precipitation; ± = trace of precipitate.

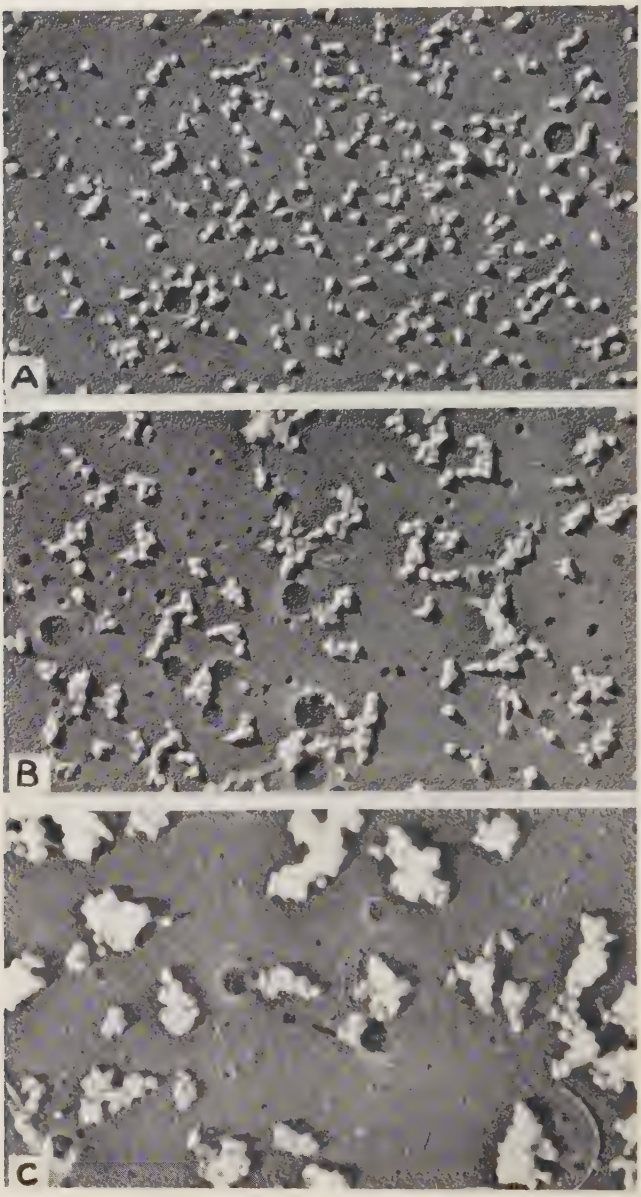

FIG. 1. Electron micrographs of virus of erythromyeloblastic leukosis precipitated from plasma with serum from chicken hyperimmunized with the virus (×14,500). A. Control, filtered plasma containing 2.8×10^{11} virus particles/ml. B. Same treated with immune serum for 1 min. at 37°C. C. Same treated with immune serum for 3 min. at 37°C.

22% of the virus was able to exert its infectious properties.

Precipitation: In Table II are shown the results of a precipitation test with a purified virus concentrate. The virus concentrate in the initial mixture, containing 17.1 x 10^{10} virus particles (per 0.4 ml total volume), was strongly opalescent, and the highest dilution showing precipitation was still visibly turbid. With complete precipitation, the supernatant fluid, after centrifugation of the reaction mixture at 900 x g for 10 minutes, was entirely clear.

Electron micrographs of the virus particles

TABLE III. Distribution of Particles and ATPase Activity of the Virus of Erythromyeloblastic Leukosis in the Supernatant and Precipitate Obtained on Treatment of Virus-Containing Filtered Plasma with the Serum from a Chicken Hyperimmunized with the Virus.

	Supernatant				Precipitate	
Serum dilution	Particle* count	% particles	ATPase* activity	% ATPase activity	ATPase* activity	% ATPase activity
	$\times 10^{11}$		γ P/min.		γ P/min.	
Control	6.13	100	71.5	100	—	—
Undil.	0.36	6	4.9	7	69.2	97
1:2	2.27	37	32.5	45	28.0	39
1:4	6.18	101	78.2	109	0.5	0.7

* Per ml final mixture of plasma and immune serum.

in plasma treated with immune serum are shown in Fig. 1. In Fig. 1A the particles appear as they occurred in the control untreated filtered plasma. The concentration of the plasma was the same as that in the test mixtures, and the content of virus particles was 2.8 x 10^{11} per ml. A picture of the particles of the plasma exposed to the immune serum for 1 minute, Fig. 1B, reveals the beginning formation of small clumps. A sample of the same mixture taken after a total of 3 minutes showed, in Fig. 1C, the large coherent masses constituted, obviously, of the particles seen in the electron micrograph of Fig. 1A. More lengthy action of the immune serum resulted in progressively larger agglomerates of the particles.

Precipitation of ATPase Activity: As indicated above, investigation has revealed(12-14) an intimate association with the virus particles of the enzymatic activity to dephosphorylate adenosine triphosphate. It was of interest to determine the relationship of the enzyme activity to the virus particles specifically precipitated with the chicken immune serum. The results of an experiment for this purpose are summarized in Table III. A series of mixtures of dialyzed virus plasma was made with 3 different amounts of immune serum as indicated. The control plasma with no immune serum and of the same volume and salt concentration as the test mixtures contained a total of 6.13 x 10^{11} virus particles. Counts could be made only on the particles remaining in the supernatant fluids after precipitation since the clumped virus could not be redispersed. This, however, did not prevent measurement of the ATPase activity of the virus in the sedimented precipitates after 2 washings with physiological saline solution. It is seen that the particles and ATPase activity remaining in the supernatant fluids were quantitatively proportional. Furthermore, in each mixture the sum of the ATPase activity of the supernatant fluid with that of the precipitate was identical, within experimental limits, with the activity of the control preparation.

Discussion. The results of the experiments demonstrate clearly the capacity of the chicken to respond immunologically to the virus of erythromyeloblastic leukosis by the production, contrary to earlier thought(23), of typical antiviral humoral antibodies which neutralize and precipitate the agent. As a consequence it is evident that the properties of the virus as an antigen do not differ in principle from those of other filterable agents. The response is readily and consistently stimulated by a simple sequence of injections of virus-containing material. It is probable that previous difficulties(24,25) in the induction of these immune bodies in the chicken have been dependent for the most part on the lack of sufficient virus in the doses employed. The virus content of the plasmas from diseased chicks varies through at least a 1,000-fold (12,14), and probably much wider, range. Only a few fully diseased chicks, not more than 5 to 10%, provide plasma containing virus particles of the order of 10^{11} to 10^{12}/ml, and only these are useful in the immunizing procedure. Such birds were selected in the present work from large numbers with the disease by micro tests of ATPase activity, without which consistent results would have

been highly improbable. It is likely, also, that the infrequent occurrence(24,25) of neutralizing antibodies in the natural disease is related to small amounts of extracellular virus available to the site of antibody formation. Most diseased chicks of high plasma-virus content die quickly, and it would appear that many of those experiencing retrogression fail to develop the physical amounts of the agent significant for antibody induction. This does not mean that experience with the virus does not result in the consistent induction of acquired resistance; the opposite is true(19), though the basis for much of it must be one unrelated to circulating antibodies(25). Quantitative measurement of the neutralizing capacity of the immune serum is greatly dependent not only on an accurate method for virus titration but on an optimum balance between virus and immune serum. The sensitivity of the precipitin reaction was enhanced to practical levels by the use of salt solution in high concentration(20).

The experiments provide the final specific evidence needed to identify, unequivocally, the particulate material as the virus and as the bearer of the enzyme dephosphorylating adenosine triphosphate and, as recently observed(26), inosine triphosphate. The electron micrographs of Fig. 1 are particularly informative in revealing the characteristic particles as the sole particulate material constituting the precipitated agglomerates. It is still not possible to employ the findings in the resolution of the ultimate question of whether the enzyme is an intrinsic constituent of the virus or simply adsorbed on the particle. Nevertheless, it is certain that, for practical purposes, the relation of the enzyme to the particles appears no less specific than that of the property of infectivity. In its response to the precipitative action of the immune serum, without significant inhibition or neutralization of its activity, the ATPase does not differ from many other enzymes(27). A difference is seen, however, from the mucinase(28) of the influenza(29,30) and other hemagglutinative viruses which is neutralized by specific immune serums(31). It should be remembered that the ATPase of the virus of erythromyeloblastic leukosis is but a part of a relatively large particulate entity of which components other than the enzyme may constitute the principal, if not the entire, antigenic material of the particle. Thus, while the enzyme is precipitated inseparably along with the virus, there is no direct evidence of specific involvement of the enzyme moiety in the immunological reaction.

A most significant consideration is that concerned with the value of these specific chicken antiserums in further studies on the virus of erythromyeloblastic leukosis and its antigenic relationships to the agents of other forms of the leukosis complex. In addition, the behavior of this tumor virus is indicative of the possibility of similar findings with the other analogous agents or agent-variants under suitable conditions.

Summary. Chickens have been hyperimmunized with formolized concentrates of the virus of avian erythromyeloblastic leukosis together with the untreated plasma of diseased birds containing the agent in high concentration. The resulting immune serums strongly neutralized the infectious properties of the virus and precipitated the characteristic virus particles as observed macroscopically and corroborated by electron micrographs of the precipitates. Precipitation of the virus particles was associated with proportional precipitation of the enzyme activity of the virus to dephosphorylate adenosine triphosphate. The findings constitute the critical specific criterion needed to establish the particles as the virus and the enzyme as a component inseparable, by all methods yet tried, from these virus particles.

1. Eckert, E. A., Beard, D., and Beard, J. W., *J. Nat. Can. Inst.*, 1951, v12, 447.

2. ———, with an appendix by B. R. Burmester, *ibid.*, 1953, v13, 1167.

3. Jungherr, E., Doyle, L. P., and Johnson, E. P., *Am. J. Vet. Res.*, 1941, v2, 116.

4. Jungherr, E., In Biester, H. E., and Schwarte, L. H., Diseases of Poultry, 2ed., The Iowa State Press, Ames, Iowa, 1948.

5. Kabat, E. A., and Furth, J., *J. Exp. Med.*, 1940, v71, 55.

6. Furth, J., and Kabat, E. A., *ibid.*, 1941, v74, 247.

7. Kabat, E. A., and Furth, J., *ibid.*, 1941, v74, 257.

8. Furth, J., PROC. SOC. EXP. BIOL. AND MED., 1932, v29, 1236.

9. Rothe Meyer, A., and Engelbreth-Holm, J., *Acta Path. et Microbiol. Scand.,* 1933, v10, 261.

10. Sharp, D. G., Eckert, E. A., Beard, D., and Beard, J. W., *J. Bacteriol.,* 1952, v63, 151.

11. Sharp, D. G., and Beard, J. W., *Biochimica et Biophysica Acta,* 1954, v14, 12.

12. Mommaerts, E. B., Sharp, D. G., Eckert, E. A., Beard, D., and Beard, J. W., *J. Nat. Can. Inst.,* 1954, v14, 1011.

13. Sharp, D. G., Mommaerts, E. B., Eckert, E. A., Beard, D., and Beard, J. W., *ibid.,* 1954, v14, 1027.

14. Eckert, E. A., Sharp, D. G., Mommaerts, E. B., Reeve, R. H., Beard, D., and Beard, J. W., *ibid.,* 1954, v14, 1039.

15. Mommaerts, E. B., Beard, D., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 479.

16. Green, I., Beard, D., Eckert, E. A., and Beard, J. W., *ibid.,* 1954, v85, 406.

17. Waters, N. F., *Poultry Sci.,* 1945, v24, 259.

18. ———, *ibid.,* 1951, v30, 531.

19. Eckert, E. A., Beard, D., and Beard, J. W., *J. Nat. Can. Inst.,* 1954, v14, 1055.

20. Goodman, M., Wolfe, H. R., and Norton, S., *J. Immunol.,* 1951, v66, 225.

21. Sharp, D. G., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 75.

22. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.,* 1925, v66, 375.

23. Jármai, K., *Proc. of 12th Internat. Vet. Cong.,* 1934, v3, 235.

24. Olson, C., *Mass. Agric. Exp. Sta.,* 1940, Bull. No. 370, 1.

25. Storti, E., and Mezzadra, G., *Folia Hematologica,* 1940, v64, 30.

26. Green, I., and Beard, J. W., *J. Nat. Can. Inst.,* in press.

27. Marrack, J. R., In Sumner, J. B., and Myrbäck, K., *The Enzymes,* 1950, v1, Part 1, 343.

28. Eckert, E. A., Lanni, F., Beard, D., and Beard, J. W., *Science,* 1949, v109, 463.

29. Hirst, G. K., *ibid.,* 1941, v94, 22.

30. McClelland, L., and Hare, R., *Canad. Pub. Health J.,* 1941, v32, 530.

31. Hirst, G. K., *J. Exp. Med.,* 1942, v76, 195.

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Does Monozol® Stimulate Elaboration of Pituitary Gonadotrophin?* (21531)

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The monobenzyl ether of diethylstilbestrol (Monozol)® has been suggested as a stimulator of pituitary gonadotrophic activity in man(1).

We administered Monozol in doses of 1.5-6.0 mg/day to 8 patients with proved deficiency of gonadotrophin. Assays for pituitary gonadotrophin in the urine were negative at 5 mouse uterine units(2) per 24 hours before treatment in each case (Table I).

The only clinical manifestations during treatment were estrogenic effects. Vaginal cornification occurred in all of the women. The 2 men showed mild gynecomastia and hyperpigmentation of the nipples and areolae. There was no evidence of androgenic activity (erections, libido, growth of genitalia or growth of facial or body hair), which would be expected had there been stimulation of pituitary gonadotrophic activity.

The gonadotrophic fraction of the urine was concentrated by adsorption to kaolin. Initially the eluates were injected into mice for assay without further treatment(3). The eluates obtained from the urine of the patients during treatment with Monozol caused enlargement of the uterus of intact immature mice. While the uterine response could have been consistent with a gonadotrophic effect, the complete lack of clinical evidence for pituitary stimulation suggested that the uterine stimulation might be due to estrogenic contaminants. To test this point we injected into ovariectomized mice the eluates which

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TABLE I.

Sex	Age	Diagnosis	Dose (mg) & duration of treatment with Monozol	Vaginal smear		Gonadotrophin			Comments
				Initial	Experimental	Initial (mu)	Experimental (mu)		
♀	19	Hypogonadotrophic eunuchoidism	3.0, 4 mo 6.0, 7	Atrophic	Cornified	−5	+ 5*	(ke)	Well-being; withdrawal bleeding
♂	36	"	3.0, 2	—	—	−5	+20,† −40	(ke)	Gynecomastia; weakness; malaise
♀	22	"	1.5, 7 3.0, 4	Atrophic —	Cornified "	−5	+20,‡ +10*	(ke)	Nausea; malaise; withdrawal bleeding
♂	25	Hypophyseal infantilism	3.0, 4	—	—	−5	+ 5,† −20	(ke)	Gynecomastia; fatigue; malaise
♀	29	Craniopharyngioma with panhypopituitarism	3.0, 9	Atrophic	Cornified	−3	− 5	(ap)	Withdrawal bleeding
♀	31	Chiari-Frommel(6) syndrome	6.0, 9	"	"	−5	+ 5*	(ke)	—
♀	40	"	3.0, 2	"	"	−5	+20†	(ke)	Suppression of lactation; mastodynia; malaise
♀	40	Postpartum hypopituitarism	3.0, 7	"	"	−5	+20,* +80†		Withdrawal bleeding

* Uterine growth obtained in castrate mice † Not tested in castrated mice. ‡ No uterine growth in castrated mice.
ke = kaolin eluate; ap = acetone ppt.

had stimulated uterine growth in intact mice (5 eluates from the urine of 4 patients). Four of the specimens produced uterine enlargement in the absence of gonads. It was apparent that this uterine growth was not due to gonadotrophic substances.

To eliminate false positive reactions for gonadotrophin due to estrogenic contamination, we used a modification of the assay technic. When the eluates were treated with acetone at pH 4 and the precipitates washed with ether, as recommended by Dekanski(4), estrogenic contaminants were completely removed.

Thus, the administration of Monozol not only produced estrogenic effects in the recipients, but also the excretion of estrogenic metabolites in their urine. Jungck and Brown (5) have also pointed out that Monozol exerts an appreciable estrogenic activity in women.

Summary. Administration of Monozol, 1.5 - 6.0 mg/day, to 8 hypogonadotrophic human subjects resulted in estrogenic phenomena and the excretion of estrogen in the urine, but did not induce elaboration of pituitary gonadotrophin.

1. Werthessen, N. T., and Gargill, S. L., *J. Clin. Endocrinol.,* 1952, v12, 169.

2. Klinefelter, H. F., Albright, F., and Griswold, G. C., *ibid.,* 1943, v3, 529.

3. Scott, L. D., *Brit. J. Exp. Path.,* 1940, v21, 320.

4. Dekanski, J., *ibid.,* 1949, v30, 272.

5. Jungck, E. C., and Brown, W. E., *J. Clin. Endocrinol.,* 1953, v13, 730.

6. Forbes, A. P., Henneman, P. H., Griswold, G. C., and Albright, F., *ibid.,* 1954, v14, 265.

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Effect of Levallorphan Tartrate Upon Toxicity of Morphine.* (21532)

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The effect of nalorphine upon toxicity of morphine has been reported recently(1). This report states that in mice, nalorphine produced observable protection against certain doses of morphine. As the dose of morphine increased the optimum dose of nalorphine decreased. As the dose of nalorphine increased above this optimum, additive toxicity developed. In the range of doses producing this additive toxicity, nalorphine and morphine were equally toxic. Apparently levallorphan is similar in action to nalorphine. It neutralizes many actions of morphine (*i.e.*, inhibition of respiration, analgesis, inhibition of dilatation reflex of guinea pig's small intestine, and cerebral excitement in cats)(2). These authors were unable to demonstrate protection against morphine in mice and rabbits upon administration of levallorphan.

The present experiments were designed to demonstrate such an antagonism in mice, to determine the optimum protective dose, and to examine the combined toxicity of levallorphan and morphine.

Method. Six hundred and twenty, standard, albino mice were used. Ten males and 10 females were selected at random for each dose combination of morphine sulfate and/or levallorphan tartrate. Animals were weighed to the nearest 0.5 g. The weights of males ranged from 16 to 22 g (mean weight of 19 g) and of females 17 to 24 g (mean weight 21 g). Dilutions of drugs were prepared in 0.9% sodium chloride solution so that the volume administered was 0.5 ml/20 g of mouse-weight. The drugs were given together under the skin of the back just to one side of midline. The doses of morphine and/or levallorphan are presented in Fig. 1.

Results. Fromherz and Pellmont(2) state that in mice morphine antagonists do not affect the lethal doses of morphine. Both previous work with nalorphine(1) and these results indicate that a definite protection is produced by either of these drugs. The antagonism produced by levallorphan, except for quantitative differences, appears identical to that produced by nalorphine. In each case the mixtures even in lethal quantities were less apt to produce the Straub tail response and convulsions than were the larger doses of morphine alone. This quieting effect was apparently more marked with levallorphan than with nalorphine.

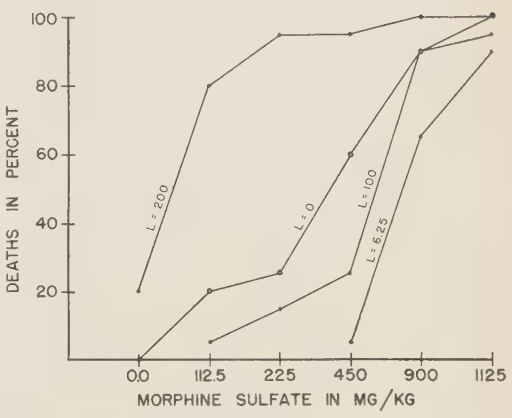

FIG. 1. No. of deaths in each group of 20 mice is plotted against doses of morphine. Curves indicate changes developing at fixed doses of levallorphan.

In Fig. 1, % of deaths is plotted against doses of morphine sulfate. Curves represent combined data from the 2 sexes at different dose levels of levallorphan. Sigmoid type curves are suggested. As the dose of levallorphan is gradually increased, the curves shift first to the right and later to the left and upward. The shift to the right indicates an increasing resistance to morphine (or protection), while the shift upwards indicates toxicity to levallorphan.

At fixed doses of morphine, the relationship between dose of levallorphan and number of

* Levallorphan (3 - hydroxy - N - allymorphinan, Hoffmann-LaRoche) was supplied by Hoffmann-La-Roche Co. I wish to thank this company for their generosity.

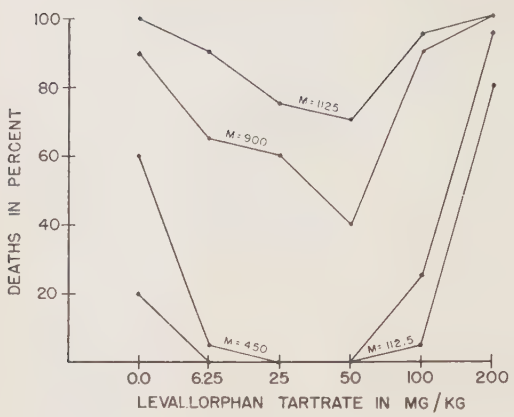

FIG. 2. No. of deaths in each group of 20 mice is plotted against doses of levallorphan. Curves represent changes occurring at fixed doses of morphine sulfate.

deaths may be observed (Fig. 2). Sigmoid curves were not obtained. The curves appear to have 2 components. The first represents protection by levallorphan against morphine. The second apparently represents the combined toxicity of morphine and levallorphan. The degree of protection produced by levallorphan was not directly proportional to the dose but decreased in intensity with each succeeding increment in levallorphan dosage. Eventually a point of maximum protection was reached beyond which toxicity was increased by increasing the dose of levallorphan.

In these mice, a dose of 50 mg of levallorphan/kg appears to have produced maximum protection against all doses of morphine. The previous report(1) states, "As the dose of morphine increased, the amount of N-allylnormorphine which could be given before this maximum was reached became less and less." This may be a true difference between nalorphine and levallorphan. It is more likely, however, that the difference lies in the experimental technic. If more gradual increases in the dose of levallorphan had been made, an inverse relationship between the dose of morphine and the dose of levallorphan producing maximum protection when given in combination might have been observed.

An equation for levallorphan-morphine combined toxicity was developed which is similar to the one presented in the previous paper(1). This equation states that Tc = 323 (L) + 46.5 (M) — 90, where Tc is the combined toxicity (% of mice expected to die), (L) is the millimoles of levallorphan/kg, and (M) is the millimoles of morphine/kg. A test for the goodness of fit for this equation to the original data (Chi square) indicated that the equation is a good characterization of the results(3). This equation suggests that levallorphan is 7 times as toxic as morphine (on a molar basis) when the 2 drugs are given together. In a similar experiment(1) nalorphine and morphine were equally toxic. In comparing the 2 experiments, levallorphan is obviously more potent than nalorphine in antagonizing the lethal effects of morphine in mice. It is probable that the potency and toxicity were parallel for these 2 drugs when given with morphine.

The LD_{50}'s for morphine at various concentrations of levallorphan were determined by the method of Litchfield and Wilcoxon(4). A summary of these findings is presented in Table I. The lines used to determine the

TABLE I. LD_{50}'s of Morphine Sulfate in mg/kg when Dose of Levallorphan in mg/kg Is Altered. Determinations were made by method of Litchfield and Wilcoxon(4).

Levallorphan	LD_{50}	f LD_{50}	S	f LD_{50}
.0	300	1.35	2.20	1.27
3.125	620	1.13	1.42	1.02
6.25	750	1.45	1.55	1.36
12.5	682	1.43	1.50	1.30
25	760	1.32	2.14	1.50
50	1000	1.17	1.56	1.27
100	521	1.27	1.98	1.27
200	58	2.40	2.80	2.90

LD_{50}'s showed no significant heterogeneity when examined by the Chi square test as recommended by these authors. Intercomparison shows only 2 of the lines to have significantly different slopes.

Conclusions. 1. Levallorphan when given to mice produces observable protection against certain doses of morphine. 2. As the dose of levallorphan is increased an optimum dose is reached. If given in doses larger than this optimum dose, an additive toxic effect between levallorphan and morphine occurs. 3. In the range of additive toxicity levallorphan is approximately 7 times as toxic as

morphine on a molecular basis.

1. Gruber, Jr., C. M., *J. Pharm. and Exp. Therap.*, 1954, v111, 404.

2. Fromherz, K., and Pellmont, B., *Experientia*, 1952, v8, 394.

3. Mainland, D., *Elementary Medical Statistics*, W. B. Saunders Co., Philadelphia, Pa., 1952.

4. Litchfield, Jr., J. T., and Wilcoxon, F., *J. Pharm. and Exp. Therap.*, 1949, v96, 99.

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Experimental Dental Caries VIII. Effect of Thyroid - Testosterone Administration on Dental Caries in Rat.* (21533)

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Desiccated thyroid added to the dry diet has been shown in a previous report(1) to reduce significantly the incidence of dental caries in rats receiving a cariogenic diet, while a significant increase in the dental caries experience was found when thiouracil was added to the diet. These data suggest that the activity of the thyroid gland may be related to the incidence of dental caries in rats. In speculating as to a possible mechanism by which such a cariogenic effect could be produced, one is impressed with the relationship between the salivary glands and dental caries as well as with the fact that testosterone increases the secretory activity of the thyroid gland(2). Previous work has shown a relationship between the gonads and the submaxillary gland(3-5), and since experiments in our laboratories have suggested a relationship between the histologic structure of the submaxillary gland and the dental caries experience(6) it seemed plausible that the effect of the thyroid on the teeth may be mediated through the salivary gland.

Since testosterone has been shown to increase the secretory activity of the thyroid gland, and due to the work of Grad and Lebond who have demonstrated the relationship between the thyroid and the submaxillary glands(7), it became of interest to determine if a synergism between testosterone and desiccated thyroid would result in a greater increased anti-cariogenic effect in rats than produced by desiccated thyroid alone.

Materials and methods. A total of 225 weanling Sprague-Dawley strain rats were divided according to initial body weight into 6 experimental groups with each composed of equal numbers of males and females. One group received desiccated thyroid in the diet (in increasing amounts from 10 to 20 mg/day calculated by food consumption and depending upon the growth of the animal) and another received the same amount of desiccated thyroid plus testosterone† (aqueous suspension 2.5 mg per week per animal injected intramuscularly). Eight weeks after the initiation of the experiment, the dose of testosterone was reduced to 1.25 mg for 5 weeks and then restored to the original amount for the remainder of the experiment). A third group received propyl thiouracil (0.1% in the diet), while a fourth group received the thiouracil diet plus testosterone at the same level as received by the thyroid-testosterone animals. A fifth group received testosterone alone and a sixth group received neither testosterone, thyroid nor thiouracil and served as controls. All of the animals were housed in raised screen cages and received the same cariogenic stock corn diet (Fluorine = 0.5 μg/g) and fluorine-low (F = 0.5 μg/ml) drinking water. The duration of the experiment was 126 days, after which time the animals were sacrificed

* This investigation supported in part by Medical Research and Development Board, Office of Surgeon General, Department of Army.

† The authors wish to thank Schering Corp., Bloomfield, N. J. for generously supplying the testosterone (Oreton-F).

TABLE I. Comparison of Dental Caries in Rats Receiving Desiccated Thyroid, Thiouracil or Testosterone.

Group	Sex	No. of rats	Final wt (g)	Dental caries: No. of lesions	Dental caries: % difference		Probability*	Skeletal analysis (ashed femur): Wt (g)	F conc. (ppm)	Total F (mg)
Thyroid	♂	13	254	4.9	−21	−20	.05	.3038	59.5	.0183
	♀	18	190	5.9	−19			.2201	83.1	.0180
Thyroid + test.	♂	14	319	3.7	−41	−40	.002	.2791	71.8	.0187
	♀	18	205	4.5	−39			.2873	53.8	.0148
Thiouracil	♂	19	180	8.9	31	25	.02	.2127	79.6	.0173
	♀	15	145	9.0	19			.1501	78.4	.0117
Thiouracil + test.	♂	16	199	9.2	34	27	.02	.2291	65.1	.0148
	♀	18	167	9.1	20			.1976	52.6	.0128
Testosterone	♂	15	291	7.4	14	3	.19	.3063	59.4	.0183
	♀	17	233	6.7	− 8			.2732	68.0	.0185
Control	♂	17	335	6.2	—			.3191	57.1	.0175
	♀	15	211	7.3	—			.2401	65.1	.0158

* Based on differences between mean No. of lesions (sexes combined) in the 6 groups, not percentage differences.

by chloroform anaesthesia, the heads removed for dental caries evaluation by methods previously described(8), and the salivary glands, thyroid glands and other organs fixed for histologic study. The femurs were also removed for fluorine analysis.

Results. The results of these experiments are seen in Table I, and corroborate the previous observation(1) that desiccated thyroid reduces the incidence of dental caries in rats while thiouracil increases the caries incidence when compared to control rats receiving neither thyroid nor thiouracil. In addition, these data clearly indicate that testosterone increases the anti-cariogenic effect of desiccated thyroid, while testosterone itself is without effect on the incidence of dental caries.

The histologic features of the thyroid glands of animals receiving thiouracil and desiccated thyroid were characteristic. The thyroids of the former groups were grossly enlarged and exhibited extreme hyperplasia of the thyroid epithelium with nearly complete absence of colloid. The latter groups of animals exhibited thyroids which were very slightly enlarged grossly. This appeared to be due to the accumulation of colloid with simple distention of the gland. The lining epithelial cells were extremely flattened indicative of their inactive state.

The amount of fluorine stored in the skeleton as evaluated by the femur-fluorine levels indicates that none of the supplements significantly affected the storage of fluorine.

Discussion. The relationship between the activity of the thyroid gland and the incidence of dental caries in rats has been confirmed and extended. These data obtained in this study indicate that administration of desiccated thyroid is associated with a reduction in dental caries while a decreased activity of the gland induced by administration of thiouracil results in a marked increase in the dental caries experience. It is an inviting hypothesis to suggest that this effect is mediated through subsequent changes in the function of the salivary glands. These conclusions are interesting in light of the work of Grad and LeBlond(7) who report that the administration of thyroxine and testosterone to control rats with testes and thyroid gland extirpation, restores to normal the size, granule content and number of cells of the serous tubule in the submaxillary gland. Furthermore, these same authors report a diminution in the size of the average diameter of the serous tubules of the rat submaxillary gland following thyroidectomy. The evidence of a synergism between this androgen and desiccated thyroid corroborates the previously published work of Burris *et al.*(2) who demonstrated histologically an increased activity of the thyroid gland under such experimental conditions. The observation that testosterone itself does not effect the incidence of dental caries corroborates previous work from these

laboratories(9). However, one might predict from the work of Burris *et al.*(2) that this androgen would in itself reduce the incidence of dental caries if its effect is to increase the activity of the thyroid gland and if the thyroid gland is directly related to caries activity. Since our experience indicates that testosterone does not alter the dental caries experience but testosterone-thyroid supplementation does, it may well be that testosterone itself does not significantly modify the function of the salivary gland, but requires a synergistic agent such as the thyroid hormone in order to maintain the physiological function of the salivary gland throughout the experiment.

Summary. Activity of the thyroid gland appears to be related to incidence of dental caries in the rat. These conclusions are based upon experimental observations that administration of desiccated thyroid reduces dental caries and thiouracil increases it. This cariogenic effect might be related to subsequent function of salivary glands, since it has been shown that when desiccated thyroid and testosterone were administered together it increases to a greater degree the anti-cariogenic effect of desiccated thyroid. This fact is in keeping with the observation that testosterone increases activity of the thyroid gland and that the thyroid gland is related to the function of the submaxillary gland.

1. Muhler, J. C., and Shafer, W. G., *Science,* 1954, v119, 687.

2. Burris, M. J., Bogart, R., and Kruegar, H., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 181.

3. Lacassagne, A., *Comp. Rend. Soc. de Biol.,* 1940, v133, 227.

4. Buillard, H., and Delsuc, P., *ibid.,* 1941, v134, 741.

5. Shafer, W. G., and Muhler, J. C., *J. Dent. Res.,* 1953, v32, 262.

6. ———, unpublished data.

7. Grad, B., and Leblond, C. P., *Endocrinology,* 1949, v45, 250.

8. Muhler, J. C., Nebergall, W. H., and Day, H. G., *J. Am. Dent. Assn.,* 1953, v46, 290.

9. Muhler, J. C., and Shafer, W. G., *J. Dent. Res.,* in press.

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Efficacy of 9 Alpha-halo Adrenal Steroids for Maintenance of Adrenalectomized Dogs.* (21534)

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Fried and Sabo(1,2,2a) first reported on the greatly enhanced glucocorticoid activity of corticosteroids in which the 9 α-hydrogen atom is replaced by halogen and their initial findings have recently been confirmed and extended. Through the courtesy of Dr. Fried, of the Squibb Institute for Medical Research, New Brunswick, New Jersey, sufficient of the fluoro-F-acetate, chloro-F-acetate and fluorocorticosterone acetate were obtained for testing their life maintenance potency and mineralocorticoid activity on adrenalectomized dogs.

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Methods. The animals had been adrenalectomized for periods ranging from 2-5 years and during this interval were subjected to numerous bouts of insufficiency and recovery on adrenal steroid therapy. In the interim between experiments they were maintained on 0.5 mg DCA in oil injected i.m. once daily. The diet was rigidly controlled and the daily amount of Na and K allowed each dog, including that in the food and drinking water, totaled 1.47 and 0.94 g respectively. The compounds tested were solubilized in 95% alcohol then diluted to 10-15% and administered s.c. in divided doses twice daily at 9 a.m. and 5 p.m. The dose was reduced by 50% at the end of each 10 day interval and

TABLE I. Maintenance of Adrenalectomized Dogs with 9 α-Halo Adrenal Steroids.

Dog No.	Dose dog/day, μg	Exp. period, days	Wt, kg	B.P., mm Hg	Blood urea N, mg %	Hb, g %	Hmct., %	R.B.C. 10^6/mm³	Blood sugar, mg %	Serum Na, m.Eq./l	Serum Cl, m.Eq./l	Serum K, m.Eq./l
					9 α-Chlorohydrocortisone acetate							
1		Initial	16.56	110	23.3	11.71	32.5	5.42	90	144.0	116.0	4.79
	110.00	10	16.56	100	23.1	11.94	34.9	6.79	87	142.0	111.1	4.71
	55.00	10	16.44	102	25.0	12.51	38.8	7.07	84	145.0	111.4	5.28
	27.50	10	15.76	83	45.4	19.24	41.5	7.21	82	134.5	107.0	7.78
	13.75	8	15.65	66	87.0	20.14	45.6	7.42	83	133.0	108.0	9.94
					9 α-Fluorohydrocortisone acetate							
2		Initial	14.74	108	15.3	11.00	32.2	6.05	81	144.5	112.8	4.49
	110.00	10	14.62	110	18.4	11.10	32.7	5.96	83	144.0	112.8	4.46
	55.00	10	14.85	109	17.9	11.59	33.0	6.19	84	145.0	114.0	4.64
	27.50	10	14.51	105	19.0	12.91	33.8	6.55	80	140.0	108.6	4.71
	13.75	10	14.51	91	22.2	15.60	38.6	7.61	80	137.0	106.0	5.30
	6.87	9	14.85	70	33.1	15.43	41.9	7.76	81	120.5	96.8	7.15
					9 α-Fluorocorticosterone acetate							
3		Initial	14.74	98	13.6	12.27	35.1	5.30	73.0	144.5	115.5	4.54
	110.00	10	14.74	110	18.2	11.94	34.0	5.20	81.5	148.5	119.2	3.90
	55.00	10	14.85	96	18.3	12.28	31.2	4.80	72.5	146.0	116.0	4.16
	27.50	10	14.51	104	25.6	12.77	29.6	4.61	82.0	152.5	115.5	4.79
	13.75	10	14.51	99	23.1	12.51	31.1	5.44	76.5	150.5	115.5	4.81
	6.85	10	13.95	99	25.9	11.19	27.1	4.23	81.5	149.0	112.8	5.61
	3.42	10	14.29	64	27.8	14.55	35.5	6.17	73.0	130.0	102.5	6.34

Italicized dosages are minimum maintenance dosages.

samples taken for blood chemical determinations. The minimum maintenance dose (M M D) was considered to be the daily amount required to keep the animals symptom-free and the blood constituents and blood pressure, little, if any changed from the normal or initial values. Tests for the fluoro-F-acetate and chloro-F-acetate generally required 30-50 days for completion, whereas the fluorocorticosterone acetate necessitated 60 days before marked signs of adrenal insufficiency appeared. The methods employed for the various determinations have been repeatedly referred to in earlier communications and hence are omitted here.

Results. 1. 9 *Alpha Chlorohydrocortisone Acetate.* This material has been tested on rats(3,4,5), dogs and Addisonians(6,7) and shown to possess both mineralo and glucocorticoid activity in high degree. The experiments recorded here were conducted on 5 adrenalectomized dogs; the MMD was 55-110 μg/dog/day since 2 animals were maintained on 55 and 3 on 110 μg. The dose upon which the dogs developed unmistakable symptoms of insufficiency was considerably less than the MMD and varied between 13.75 and 55 μg/day. The pertinent data for one typical example are presented in Table I, dog 1.

2. 9 *Alpha Fluorohydrocortisone Acetate.* This compound according to brief reports which have appeared, is said to be much more potent than DCA and cortisone with respect to both salt retaining and glycogen deposition activity when used on either experimental animals(2,4,6), or the Addisonian patient(6). The present experiments confirm these reports and present in detail the alterations in blood pressure and blood constituents accompanying its use in 3 dogs. The essential data obtained from study of a representative case are given in Table I, dog 2.

The MMD for the 3 animals was 27.5 μg/dog/day. Although lower doses, *e.g.*, 13.75 and in 2 cases 6.87 μg/day proved capable of sustaining the animals, they were inadequate to prevent the appearance of the initial signs of impending adrenal failure. The early indications of adrenal insufficiency in the dogs receiving these 9 Alpha-halo derivatives of hydrocortisone are: (1) a decline in the mean arterial pressure; (2) a rise in blood urea nitrogen; and (3) elevation of the serum K. Not all of these changes may occur simultaneously nor are they invariably accompanied by alterations in the serum Na and Cl

TABLE II. Effect of 3 mg/kg/Day of 9 α-Fluoro-F upon Water and Electrolyte Excretion.

Wt,,kg	Exp. period, days	Urine electrolytes, avg m.Eq./24 hr Na	Cl	K	Avg urine vol, cc/day	Avg water intake, cc/day
16.27	1- 6	47.7	53.1	16.5	248	375
16.33	7-13*	30.2	33.3	21.2	628	881
15.65	14-20*	80.6	83.7	27.1	4786	4865
14.79	21-25	83.4	92.6	11.0	2290	2311
14.21	26-30	54.8	57.4	16.8	286	442

* 9 α-Fluoro-F administered orally.

when the test material possesses marked Na-retaining properties. One animal in the group suddenly collapsed and died before restorative measures could be instituted. This dog was receiving 6.87 μg of fluoro-F-acetate and but a few hours previous to death had shown no gross symptoms. However, the blood pressure had declined from 120 to 97 mm Hg and the serum K was 6.98 m-eq/l. It was considered not unlikely that death was due to a rapidly developing K intoxication, although no actual determinations for this cation were made. The results of the tests showed that the fluoro-F-acetate was definitely more potent for maintaining normal health and vigor in adrenalectomized dogs than was the chloro-F-acetate. The MMD for the latter compound was considerably higher.

Enough of the fluoro-F-acetate was available to study the effects induced by overdosage in adrenalectomized dogs. Prolonged dosage with large amounts of cortisone induces a diabetes-insipidus-like syndrome in both rats and dogs(10,11,12). Likewise oral administration of 3 mg/kg/day of the free alcohol of the fluoro compound for 14 days also exerts a profound effect upon water intake and output. The maximum urine volume obtained for any one 24 hour period was 7125 cc in one of the 2 animals studied, and 4140 cc for the other dog. Marked edema of the hind limbs and scrotum also developed in one, but both animals exhibited attacks of muscle tremors resembling tetany which seemed to be associated with low values for serum K. The edema and tetany-like symptoms disappeared shortly after discontinuing steroid administration. However, after stopping the injections it required 9-10 days for the abnormal diuresis to subside. This is a brief period compared to the 40 days required for complete restoration of the water balance to normal following parenteral use of cortisone acetate suspensions. Table II shows data obtained from one representative dog.

3. 9 *Alpha Fluorocorticosterone Acetate.* Only a small amount of this steroid was available but owing to the extreme potency of the material it was sufficient for testing 4 dogs. The results in all four cases were closely similar and clearly indicated that the compound was the most potent of the 9 alpha-halo corticosteroids reported to date. The MMD was found to vary between 6.5 and 13.75 μg/dog/day. However, the daily dose at which symptoms of insufficiency appeared was 3.42 μg. These dose levels are practically identical with those recorded in earlier communication from this laboratory(8,9) both for the MMD and the failing dose of aldosterone. The data are presented in Table I (dog 3) and it should be noted that the serum electrolytes did not alter appreciably until the daily dose of fluorocorticosterone was reduced to 3.42 μg. Sixty days were necessary for completion of the tests owing to the potency of the compound with resulting slow attainment of the MMD and appearance of obvious symptoms of insufficiency.

Discussion. The data illustrate a fact which has been commented upon in earlier studies(13) *viz.*, that slow reduction in dosage of certain potent Na-retaining steroids over intervals of 30-50 days may lead to accumulation of K in the serum sufficient to precipitate severe cardiac episodes and collapse. Thus it seems probable that in low dosage renal elimination of K is much less rigidly controlled by adrenal steroids than is the Na.

The MMD of aldosterone for the long term adrenalectomized dog was found to vary between 6.25 and 12.5 μg whereas the minimum requirement for DOC was 125-250 μg(8,9). According to these data fluorocorticosterone is practically equivalent in potency to aldosterone and hence approximately 20 times more potent that DOC in its ability to keep the animals symptom-free. The fluoro-F-acetate is also much more potent than DOC but has only about one-fourth to one-half the activity

TABLE III. Comparative Activity of Adrenal Steroids on Life Maintenance of Adrenalectomized Dogs.

Compound	Activity (DOC = 1)
DOC	1
Aldosterone	20
Hydrocortisone	.025-.05
Cortisone	.025-.05
Chloro-F acetate	2.27
Fluoro-F acetate	4.54-9.1
Fluorocorticosterone acetate	18.2

of aldosterone. Chloro-F-acetate although about 2.27 times more active than DOC, exhibits about one-ninth the potency of aldosterone. Table III shows the relative potencies of the various steroids as obtained from these studies on the long term adrenalectomized dog.

Summary. The 9 alpha-halo derivatives of adrenal steroids are highly potent compounds and are capable of maintaining the adrenalectomized dog free from all signs and symptoms of insufficiency at very low doses. Fluorocorticosterone was the most active compound tested and proved to be equal in potency to aldosterone. Fluoro-F-acetate was 4.5-9 times more active and chloro-F-acetate more than twice as potent as DOC. Daily doses of the fluoro-F-compound, given orally in large doses, induced marked polydipsia and polyuria accompanied by oedema of the hind legs and scrotum.

1. Fried, J., and Sabo, E. F., *J. Am. Chem. Soc.,* 1953, v75, 2273.

2. Fried, J., and Sabo, E. F., *ibid.,* 1954, v76, 1455.

2a. Fried, J. F., *J. Am. Chem. Soc.,* 1955, in press.

3. Borman, A., and Singer, F. M., *Fed. Proc.,* 1954, v13, 185.

4. Borman, A., Singer, F. M., and Numerof, R., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 570.

5. Leathem, J. H., and Wolf, R. C., *ibid.,* 1954, v86, 724.

6. Liddle, G. W., Pechet, M. M., and Bartter, F. C., *Science,* 1954, v120, 496.

7. Goldfien, A., Beigelman, P., Laidlaw, J., and Thorn, G., *J. Clin. Endocrinol. and Metab.,* 1954, v14, 782.

8. Swingle, W. W., Maxwell, R., Ben, M., Baker, C., LeBrie, S. J., and Eisler, M., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 147.

9. Swingle, W. W., Mawell, R., Ben, M., Baker, C., LeBrie, S. J., and Eisler, M., *Endocrinology,* 1954, v55, 813.

10. Winter, C. A., Ciba Foundation Colloquia Endocrinology, 1952, v4, 499, Little Brown and Co., Boston, Mass.

11. Swingle, W. W., Fedor, E. J., Ben, M., Maxwell, R., Baker, C., and Barlow, G., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 571.

12. Sirek, O. V., and Best, C. H., *ibid.,* 1952, v80, 594.

13. Swingle, W. W., Ben, M., Maxwell, R., Baker, C., Fedor, E., and Barlow, G., *Endocrinology,* 1954, v54, 698.

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Production of Experimental Lathyrism in the Rat by Two Different Beta-substituted Ethylamines. (21535)

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Crystalline glutamyl aminopropionitrile has been isolated from the seed of the flowering sweet pea (*Lathyrus odoratus*)(1-3). When it is fed to rats it produces skeletal changes typical of odoratism, the experimental lathyrism produced in rats by the feeding of sweet peas(1-3). Dasler has pointed out that the toxic crystals isolated by a method involving the use of Amberlite ion exchange resins were not glutamyl aminopropionitrile(2). He called attention to the likelihood that these crystals were the sulfate of aminopropionitrile. Preliminary evidence has indicated that aminoproprionitrile itself is indeed toxic(4). More of the latter crystals now have been prepared from sweet peas by the ion exchange method. This material has been identified with the sulfate of synthetic aminopropionitrile by

means of its infrared absorption spectrum.

Recent feeding experiments in this laboratory show that not only is synthetic β-aminopropionitrile (β-cyanoethylamine) highly effective in producing the typical skeletal changes caused by sweet peas, but similar changes can be produced in rats by the feeding of 2-aminoethanethiol (β-mercaptoethylamine).

Methods. Isolation from sweet peas of β-aminopropionitrile as crystalline sulfate. The isolation was made by a procedure which was similar to the ion-exchange method previously outlined(2). In the present preparation, 1400 g of ground sweet peas* were thoroughly extracted, first with Skelly Solve B, then with 95% alcohol. The alcoholic extract was decanted from its gummy residue and was evaporated under reduced pressure until an oily residue remained. Both residues were washed repeatedly with ether and finally partitioned between ether and water. The combined aqueous solutions were precipitated with basic lead acetate, excess lead being removed as the sulfide. The resulting solution (1300 ml) was concentrated *in vacuo* to a volume of 150 ml. This concentrate was passed through a 2.8 x 40 cm column of the strongly basic anion-exchange resin, Amberlite IRA-400 (hydroxyl form).† The effluent and washings were made acidic with H_2SO_4, then concentrated to a volume of 130 ml by vacuum distillation. This solution was now passed through a 2.8 x 40 cm column of the strongly acidic cation-exchange resin, Amberlite IR-105 (hydrogen form).†‡ The column was eluted with 1000 ml of 9% H_2SO_4. Excess sulfate was precipitated from the heated eluate by the addition of $Ba(OH)_2$ solution. The $BaSO_4$ filtrate was concentrated by vacuum distillation to 40 ml. The addition of an equal volume of 95% alcohol to the warm solution caused the immediate separation of a white sediment which was filtered off and discarded. The solution was evaporated to dryness under reduced pressure. The residue was boiled with 95% alcohol. The alcoholic solution yielded colorless crystals on cooling. The crystals were recrystallized from 95% alcohol. The dried yield of purified crystals was 1.02 g of white needles. A small amount of additional crystals was obtained from the mother liquors.

The purified crystals obtained from sweet peas by means of this isolation had an infrared absorption curve§ which was identical to that of synthetic β-aminopropionitrile sulfate. The latter compound was obtained as follows: Synthetic β-aminopropionitrile‖ was placed in a small amount of water and carefully neutralized with diluted H_2SO_4. Alcohol was added to the hot solution to incipient cloudiness. The hot solution was filtered. Long, white needles of the sulfate crystallized on cooling.

Feeding experiments. Male rats of the Sprague-Dawley strain, 26 days of age and 49 to 60 g in weight were used. The sweet pea diet was made up as described elsewhere(5), except that it contained 30% sweet peas and 45% corn starch. In the author's earlier work on the isolation of a toxic factor from sweet peas, the various extracts and concentrates were tested for their toxicity after drying them on a suitable quantity of milled Rockland Rat Diet (complete)¶ rather than incorporating them into diets made from extracted sweet peas. Toxic concentrates fed in this way caused symptoms in rats which were identical to those produced by the feeding of sweet pea diets. Based on this experience, synthetic β-aminopropionitrile‖ and β-mercaptoethylamine** were separately dissolved in small amounts of water, carefully neutralized with HCl, and the resulting solutions of the hydrochlorides were dried on suitable quantities of

* Sweet peas were generously supplied by Mr. Raymond H. Coulter of the Ferry-Morse Seed Co., Detroit.

† Rohm and Haas Co., Philadelphia.

‡ Amberlite IR-105 is no longer available. It has been replaced by other strongly acidic cation-exchange resins. The latter have not been tried in this procedure.

§ Infrared absorption spectra were determined by Albert S. Deutsch, Ill. Institute of Technology.

‖ β-aminopropionitrile was synthesized by E. D. Schilling and F. M. Strong, University of Wisconsin, to whom the author is grateful for a generous sample.

¶ Ordinary Rockland Rat Diet pellets were ground to a fine meal by a commercial miller.

** β-mercaptoethylamine purchased from California Foundation for Biochemical Research, Los Angeles.

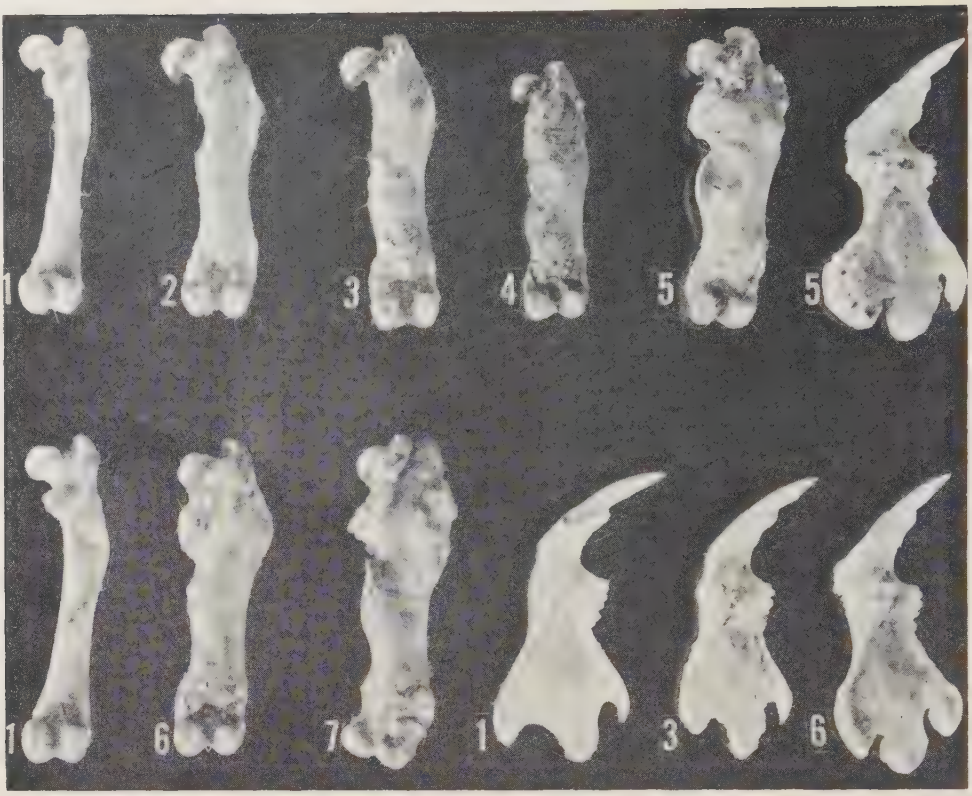

FIG. 1. Femurs and mandibles from experimental rats: (1) normal; (2) 0.25% mercaptoethylamine, killed at 21 days; (3) mercaptoethylamine, 0.25% for 14 days, 0.5% for 7 days, killed at 21 days; (4) 0.5% mercaptoethylamine, died at 12 days, this rat did not grow; (5) 0.25% aminopropionitrile, killed at 15 days; (6) 30% sweet peas, killed at 15 days; (7) 30% sweet peas, killed at 29 days.

milled Rockland Rat Diet. *Criterion of Toxicity.* As previously described (6), gross manifestations of skeletal change were used as the chief criterion of toxicity.

Results. Fig. 1 illustrates the changes that took place in the femurs and the mandibles of rats that received aminopropionitrile, mercaptoethylamine, or sweet peas in their diets.

Beta-aminopropionitrile hydrochloride at a dietary level of 0.25%, based on the free base, was markedly more effective in inducing the skeletal changes of sweet pea lathyrism than were sweet peas at a level of 30%. It seems, therefore, that the toxicity of glutamyl aminopropionitrile must lie in the aminopropionitrile portion of the molecule. Whether aminopropionitrile exists independently of the glutamyl derivative in the sweet pea is not known. It seems likely that the isolated aminopropionitrile was derived from the glutamyl derivative by hydrolysis during the course of its isolation.

Rats fed 0.5% aminopropionitrile (as the hydrochloride) did not survive beyond 17 days. In spite of very poor growth at this level, skeletal changes were maximal (not illustrated).

Beta-mercaptoethylamine also caused changes in the femurs and in the mandibles which were similar to those induced by sweet peas. Even a level of mercaptoethylamine which entirely prevented a gain in weight produced a marked malformation of the femur (Fig. 1)(4). Although preliminary experiments indicate that the mercapto-compound is at least as toxic as the nitrile when compared on the basis of its minimal lethal dose for the rat, its effect on the bones is definitely less than that of the nitrile. It is of interest to note that β-mercaptoethylamine occurs natur-

ally in the animal organism as the terminal group of the Coenzyme A molecule.

It is interesting to speculate on the significance of the above findings. It appears clear that the toxic action of these substances does not involve the calcium and phosphorus metabolism of bone directly(7). Since both of these compounds are β-substituted ethylamines, it is logical to look for a β-substituted ethylamine as the key metabolite that is affected in odoratism. Beta-ethanolamine and β-alanine are β-substituted amines which occur naturally in the body. Neither of these has any protective action against sweet peas even when fed in amounts which are large enough to inhibit growth(8). Nevertheless, the results point to the possibility that the specific metabolic defect in sweat pea lathyrism may involve the metabolism of a two- or three-carbon metabolite, probably an amine.

Summary. 1. Crystals which were isolated from sweet peas by a method which had previously yielded a toxic product were found to have the same infrared absorption spectrum as synthetic β-aminopropionitrile sulfate. 2. Synthetic β-aminopropionitrile and β-mercaptoethylamine, when fed to rats as their hydrochlorides, both produced skeletal changes similar to those caused by sweet peas. The mercaptoethylamine was the less effective in producing skeletal changes, although preliminary experiments indicate that it is at least as toxic as aminopropionitrile when judged on the basis of lethality. 3. Some of the implications of these findings are discussed.

Note added in press. Since this paper was submitted for publication, Wawzonek, Ponseti, Shepard, and Wiedenmann(9) have reported that aminoacetonitrile also produces skeletal lesions typical of odoratism. It appears, therefore, that neither the β-aminoethyl group nor the cyano group are essential for the production of this type of lesion.

1. Schilling, E. D., and Strong, F. M., *J. Am. Chem. Soc.*, 1954, v76, 2848.

2. Dasler, W., *Science,* 1954, v120, 307.

3. McKay, G. F., Lalich, J. J., Schilling, E. D., and Strong, F. M., *Arch. Biochem. Biophys.*, 1954, v52, 313.

4. Strong, F. M., personal communication.

5. Dasler, W., *J. Nutrition,* 1954, v53, 105.

6. ———, PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 485.

7. ———, *J. Nutrition,* 1954, v54, 397.

8. ———, unpublished data.

9. Wawzonek, S., Ponseti, I. V., Shepard, R. S., Wiedenmann, L. G., *Science,* 1955, v121, 63.

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Propagation of Canine Distemper Virus in Suckling Hamsters. (21536)

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Until its adaptation to the developing chick embryo(1-2), canine distemper virus was considered to be strictly host specific to the canine and musteline species. Its propagation in the chick embryo has not only extended its host range, but has resulted in the loss of its virulence for dogs, minks and ferrets, permitting its use as a live virus vaccine in these animals(3-6). A convenient method has also been provided for the detection and measurement of neutralizing antibodies in the sera of animals vaccinated against or recovering from distemper(1,7,8).

The propagation of canine distemper virus in the chick embryo may also have made possible its adaptation to suckling mice(9) and, as is reported in the present communication, its ready multiplication in the central nervous system of suckling hamsters.

Materials and methods. Virus. The virus used in this study was isolated in this laboratory from a natural case of distemper encephalitis(10) in a dog. It consisted of either vacuum dried commercial vaccine preparations, reconstituted to 40% tissue with sterile distilled water, or of freshly prepared suspen-

sions of infected chorio-allantoic membrane (CAM). All preparations represented the 42nd to 44th serial passage of the virus in the chick embryo.

Mice and hamsters. Laboratory-bred litters of Swiss albino mice and golden hamsters were used. The mice were 1 to 2 days old at the time of inoculation, and the hamsters ranged from 3 to 5 days of age. Inoculation was done intracerebrally, each animal receiving 0.02 or 0.03 ml according to size. The inoculum consisted of one of the following suspensions, prepared in beef heart infusion broth: 10, 20 or 40% reconstituted commercial vaccine, 40% infected CAM, or 20% mouse or hamster brain. All suspensions were centrifuged at 2,000 rpm for 20 minutes before inoculation. Virus titrations were made in suckling hamsters in 10-fold dilutions, care being taken that animals in each test were from litters of the same age. *Chick embryo inoculations.* These were made in White Leghorn embryonated eggs, 7-8 days of age at the time of inoculation. For propagation of the virus, a 40% infected CAM suspension was inoculated in 0.3 ml amounts on the CAM of 10 or more embryos. These were incubated at 36°C for 7 days, at which time the CAM's of surviving embryos were harvested and pooled. For virus assay, 20% hamster brain suspensions were similarly inoculated into groups of 10 embryos. CAM's of surviving embryos were examined for lesions at the end of 7 days' incubation. Lesions were scored as follows: maximum 4, somewhat less than maximum 3, and so on down to 0.5, which was assigned to embryos presenting only scattered thin streaks. Scores of embryos in the same group were added and the arithmetic mean was recorded as growth score (5th column, Table I). All suspensions inoculated into chick embryos contained enough penicillin and streptomycin to give final concentrations of 500 units of the former and 200 μg of the latter per 1 ml inoculum. *Ferrets and challenge virus.* Healthy young animals were obtained from the same source as reported earlier(2), and maintained under the same conditions. The challenge virus consisted of a 20% ferret spleen suspension infected with the Lederle stock strain of virulent distemper virus and prepared as described in a previous communication(5).

TABLE I. Details Pertaining to the Serial Intracerebral Passage of Distemper Virus in Suckling Hamsters.

Passage No.	Age of litters (days)	Incubation (days)	Ratio of infection	Growth score on CAM*	Suckling hamster titer
1	3	7	5/ 5†		
2	3	6–7	11/11	1.3	
3	3	6	13/13		
4	3	4	4/ 4	1.1	
5	4	6	7/ 7	.8	$10^{-4.0}$
6	3	4	16/16	.7	
7	3	4	13/13		
8	3	4–6	15/15	1.3	
9	3	4	5/ 5		
10	3	4–5	14/14	1.0	
11	3	4–5	10/10		
12	4	5	4/ 4	2.3	
13	5	5	5/ 5	2.1	$10^{-3.29}$
14	5	5	11/11	1.2	
15	3	4	8/ 8	1.0	
16	3	4	17/17	2.1	

* See text for definition of growth score.
† Animals either dead of infection or sacrificed when moribund, over No. of animals inoculated.

Normal and immune dog sera. These were derived from a dog as described in an earlier experiment(6). Following withdrawal of a normal blood sample, the dog was given subcutaneously 2 ml Avianized ® Canine Distemper Vaccine. After a 70-day observation period, during which the animal remained healthy, with normal temperature readings, leucocyte counts and blood sedimentation rates, it was challenged intracerebrally with the Snyder Hill strain of virus(6). The second bleeding was obtained 40 days after challenge. During the interval, the dog remained perfectly normal, whereas unvaccinated control animals died in convulsion following the same challenge. The serum was separated from the clot of each of the two bleedings at the time of withdrawal and stored in the refrigerator until used.

Serum neutralization tests. The qualitative test was made by mixing equal volumes of either normal or immune dog serum with 20% hamster brain suspensions. The serum samples were inactivated at 56°C for 30 minutes before mixing with the virus. As an additional control, equal volumes of 20% infected

® Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

hamster brain and broth were mixed. The set of 3 mixtures was held at room temperature for one hour before inoculation into hamsters of 1 or 2 litters per mixture. The animals were observed for sickness and death over a period of 21 days. The quantitative test was in every respect similar to the one above, except that 3 sets of 10-fold dilutions of the 20% hamster brain suspension were made in broth before mixing each dilution with equal volumes of either undiluted normal serum, undiluted immune serum or broth.

Experimental. Attempted adaptation of chick embryo propagated virus in suckling mice. Twelve different batches of commercial vaccine, including the one reported by others(9) as having been successfully adapted to mice, were reconstituted and inoculated each into mice of 2 to 4 litters in the form of 10, 20 or 40% suspensions. In addition, a freshly prepared 40% infected CAM suspension, representing the 44th chick embryo passage, was also inoculated into mice of 4 litters. The experimental animals were carefully watched for symptoms over a period of 21 days, and those showing the slightest deviation from normal were sacrificed and their brains harvested for further passages in baby mice. Animals evidenced either no symptoms at all on the 1st passage or, in a few cases, slight excitation. Of the 12 vaccine batches, 2nd blind passages were made with 6. Second passage mice showed still less deviation from normal than those of the first passage. Their brains were, nevertheless, harvested around the 12th day, and 3rd passages made. Although no unusual symptoms were noted, 3 lines were chosen for a 4th passage, in which the animals were sacrificed 14 and 19 days after inoculation. Hyperexcitability was seen in some mice on the 16th day, and a 5th passage was made with that line, but the 5th passage animals remained normal over the 21-day observation period. Following failure to establish the virus, mouse passages were discontinued after 5 transfers in favor of the more promising hamster inoculations.

Serial transfer of chick embryo propagated virus in suckling hamsters. In contrast to the slight success obtained in suckling mice, propagation of the virus in suckling hamsters started with the very first passage. A 40% infected CAM suspension representing the 44th chick embryo passage was inoculated in 0.02 ml volumes into hamsters of 2 litters. Some of the animals were found dead on the 7th day, and the survivors showed definite signs of illness. The animals moved slowly or were completely apathetic, most of them lying on their sides, their heads slightly bent forward between their front legs. When disturbed they attempted to walk away slowly with uncoordinated movements, often rolling on their backs as a result of the effort. These sick animals were sacrificed, their brains removed aseptically and made into a 20% suspension. Details pertaining to the serial transfer of this material through 16 passages in hamsters are summarized in Table I. Invariably, animals came down at each passage level with symptoms similar to those just described. The time lapse between inoculation and development of symptoms seemed to decrease gradually as passages progressed, going from 7 days at the 1st passage to 4 days at the 16th. All of the several passage levels which were assayed on the CAM of 7-8-day embryos produced lesions undistinguishable from those associated with chick embryo adapted distemper virus. These lesions, which diminished in intensity through the 6th passage, became gradually more marked at subsequent levels. Virus representing the 5th and 13th hamster passages was titrated in suckling hamsters and gave titers of $10^{-4.0}$ and $10^{-3.29}$ respectively. These results did not correlate with the growth score on the CAM, but the number of titrations is too small to permit generalization. It might be worth mentioning here that, unlike the attempts to

TABLE II. Results of Qualitative and Quantitative Serum Neutralization Tests.

Hamster passage	Tests	Infected hamster brain suspension or dilution plus Immune dog serum	Normal dog serum	Sterile broth
11	Qualitative (mortality ratio)	5/17	10/10	9/9
13	Quantitative (LD_{50} titers)	$<10^{-1.0}$	$10^{-3.2}$	$10^{-3.3}$

adapt the chick embryo virus directly to suckling mice, the hamster adapted virus was readily propagated in one-day-old mice beginning with the second hamster passage. However, serial passages in one-day-old mice became possible only beyond the 4th hamster passage.

Identification of the hamster propagated virus. This was accomplished by: 1) serum neutralization test in suckling hamsters and 2) immunization of ferrets with the hamster propagated virus.

1) *Serum neutralization tests.* These were performed according to the technic described under *Materials and methods.* A qualitative test was done with the 11th hamster passage virus, and a quantitative test was set up with the 13th passage brain suspension. The results, summarized in Table II, unequivocally identify the hamster propagated agent as canine distemper virus.

2) *Ferret immunization test.* Twenty per cent infected brain suspensions of the 4th, 7th and 10th suckling hamster passages were each inoculated intraperitoneally in 1 ml amounts into 2 distemper-susceptible ferrets. The animals were observed for 16 days, during which time they remained normal and healthy in appearance. They were then challenged with virulent distemper virus, also intraperitoneally, each animal receiving 1 ml of a 20% infected ferret spleen suspension, or about 1,000 lethal doses. The ferrets remained normal for a 30-day observation period. In contrast, two control animals which received the same challenge dose of virus on the same day developed clinical distemper and died, one on the 13th and the other on the 14th day following inoculation.

Discussion. The propagation of chick embryo adapted distemper virus in suckling hamsters by intracerebral passage constitutes a further example of the "genetic plasticity" of viruses. An agent which for a long period was considered strictly specific for members of the canine and musteline families was, in the course of a relatively short time, successfully adapted to grow in the chick embryo, the suckling mouse and the suckling hamster. The sequence of adaptation of this virus to these different hosts is interesting: numerous attempts to transfer the virus directly from the dog or the ferret into rodents have not been successful, whereas little difficulty was encountered following its adaptation and modification in the chick embryo. It is also noteworthy that the return of the chick embryo virus into mice and hamsters, both mammalian hosts, did not bring about reversion to virulence for the ferret and, at least within the number of passages tested, caused no detectable alteration in its antigenic makeup. The propagation of distemper virus in both suckling mice and hamsters should constitute a valuable additional tool in the hands of the research worker, to whom a quantitative serum neutralization test is made available.

Summary. The Lederle strain of chick embryo adapted distemper virus was propagated in suckling hamsters through 16 serial transfers. The virus elicited a lethal infection in this host in 4-7 days following inoculation. Proof of the identity of the hamster-propagated agent was derived from serum neutralization tests in hamsters and from the successful immunization of ferrets.

The authors are indebted to Mrs. Eleanor Osborn for her assistance in preparation of the manuscript.

1. Haig, D. A., *Onderstepoort J. Vet. Sci. and Animal Ind.*, 1948, v23, 149.

2. Cabasso, V., and Cox, H. R., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 246.

3. Cabasso, V. J., Burkhart, R. L., and Leaming, J. D., *Vet. Med.*, 1951, v46, 167.

4. Cabasso, V. J., Stebbins, M. R., and Cox, H. R., *Cornell Vet.*, 1953, v43, 179.

5. ———, *Vet. Med.*, 1953, v48, 147.

6. ———, *Cornell Vet.*, 1954, v44, 153.

7. Belcher, J., Master's Thesis, Univ. of Wisconsin, 1951.

8. Baker, G. A., Gorham, J. R., and Leader, R. W., *Am. J. Vet. Res.*, 1954, v15, 102.

9. Morse, H. G., Chow, T. L., and Brandly, C. A., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 10.

10. Cabasso, V. J., and Cox, H. R., *Cornell Vet.*, 1952, v42, 96.

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Estimation of Thyroid Hormone Secretion Rate of Intact Rat.*† (21537)

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That administration of exogenous thyroxine will reduce both thyroidal I^{131} uptake(1) and output(2) suggests the possibility of measuring thyroid secretion rate indirectly by determining amount of thyroxine required to suppress turnover of I^{131} by the thyroid. Indeed, Perry(3) reported that when rats are first given I^{131}, and different groups are then given graded doses of thyroxine, inhibition of thyroidal I^{131} output during a 48-hour period is proportional to dosage of thyroxine administered. He proposed this as a thyroid assay method.

In our research the response of the rat thyroid to dosages of thyroxine that were increased progressively at intervals in the same individuals, instead of varying dosages between groups, was explored. The procedure developed permits estimation of thyroid secretion rate in smaller groups of animals than required by earlier methods. It also points to the possibility of estimating thyroid secretion rate of individual animals.

Methods. Adult albino rats obtained from Carworth Farms, were maintained on a diet consisting of yellow corn meal 140 g, ground whole wheat 100 g, whole milk powder 80 g, linseed oil meal 40 g, alfalfa leaf meal 24 g, brewer's yeast 12 g, and iodized salt 4 g. The rat laboratory was maintained at temperature of 74 $\pm$ 1°F. In each experiment rats of only one sex and approximately same age and size were used. Each rat was injected intraperitoneally with 30-40 μc of carrier-free I^{131} as NaI. Forty-eight to 72 hours were allowed for fixation of I^{131} by the thyroid and urinary elimination of excess isotope. External thyroid counts were taken at the end of this period and at appropriate intervals thereafter, at constant geometry, by use of heavy lead counting table similar to that described by Albert(4). Either a bismuth cathode gamma counting tube (RCL, Mark 1 Model 13) or a scintillation counter (Nuclear Instrument and Chemical Corp., Model DS-1) was used. Prior to counting, each rat was injected intraperitoneally with sodium pentobarbital (30 mg/kg). In addition, ether was given by inhalation when necessary to effect complete immobilization. Counts were read on a count rate meter (Nuclear, Model 1615-B) set at 2% level of counting error. In each case the thyroid region was centered over the 13 mm tapered opening in the counting table and the rat shifted about slightly until maximum counting rate was attained. Body background counts were taken over the epigastric region. Thyroid counts were corrected by one-half the body background plus the general background by formula established by Wolff(2) and also for physical decay. The

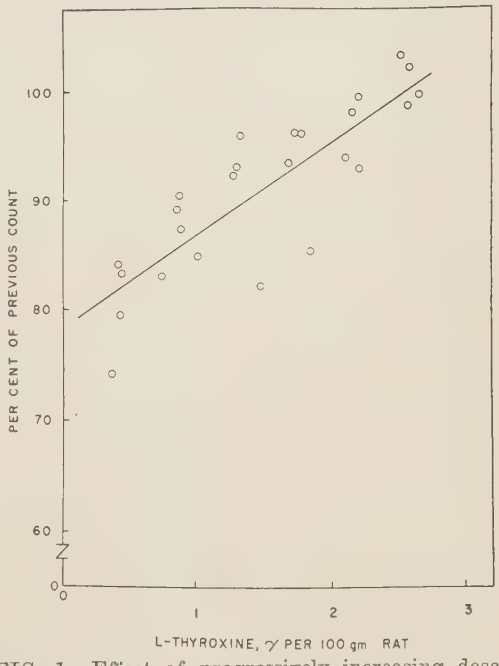

FIG. 1. Effect of progressively increasing doses of thyroxine in individual rats on retention of I^{131} in their thyroids.

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† We are indebted to Carolyn Mayotte for technical assistance and to Dr. H. A. Henneman for suggestions.

L-thyroxine‡ used as a standard was first purified by washing with absolute alcohol and then recrystallizing alternately as monosodium salt and free acid until a homogeneous crop of white crystals was obtained(5). Solutions for injection were prepared by dissolving thyroxine in distilled water with the aid of minimum amount of N/10 NaOH.

Results typical of the new procedure are shown in Fig. 1. In this experiment, 4 female rats with average body weight of 240 g were injected intraperitoneally with 40 μc of I^{131}. Seventy-two hours later an initial thyroid count was taken Each rat was then injected with 1 μg of L-thyroxine daily for 2 consecutive days and external thyroid counts were taken on third day, 48 hours after initial count. Thyroxine dose was increased progressively at 2-day intervals, a thyroid count taken before each increase in dosage. When the thyroid counts are expressed as percentage of the preceding count and plotted against thyroxine dosage, the retention of I^{131} by the thyroid increases progressively with increasing thyroxine dose until 100% point is reached and then levels off. Statistical treatment yielded a highly significant correlation coefficient of 0.843 between thyroxine dose and per cent of preceding count. A regression line was fitted to the ascending portion of curve by the method of least squares, resulting in prediction equation, $y = 78.364 + 8.435x$. If the per cent of preceding count (y) is taken as 100, the estimated thyroid secretion rate (x) is 2.56 μg/100 g body weight daily.

Highly significant correlation coefficients of 0.970, 0.937, 0.951 and 0.919 were obtained when the data for the 4 individual rats were treated separately. The estimated thyroid secretion rates of individual rats, computed from the regression equation were 2.51, 2.41, 2.29 and 3.05 μg/100 g body weight, with an average of 2.56 μg. In this experiment the thyroxine dosage increments were spaced closely enough together so that 6 points were obtained for each rat on the ascending portion of the curve.

‡ Kindly supplied by Glaxo Co., Greenford, Middlesex, England.

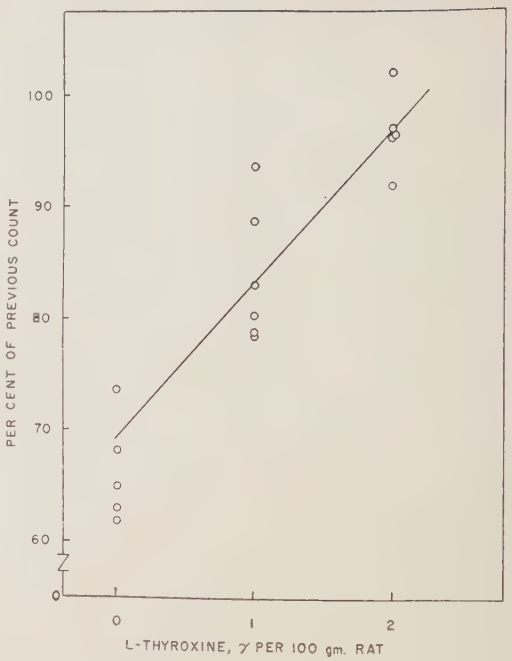

FIG. 2. Effect of wider increments of thyroxine dosage in individual rats on retention of I^{131} in their thyroids.

Fig. 2 shows the results obtained with wider spacing between thyroxine dosages. This experiment included 6 female rats averaging 246 g in body weight. The procedure was the same except that at 48 hour intervals following injection of I^{131} two external thyroid counts were taken to establish normal output rate. Thyroxine was then given on 2 successive days at levels of 1 and 2 μg/100 g body weight, counts also taken at 2-day intervals. When the data for all rats is included, there is a highly significant correlation of 0.902 between thyroxine dose and per cent of preceding counts. The estimated thyroid secretion rate was 2.21 μg L-thyroxine/100 g body weight. Data for individual rats also yielded high correlation values, but they were not statistically significant because of the small number of points for each rat. It appears, therefore, that reliable values can be obtained for individual rats only if there are not less than 5 to 6 points on the ascending part of response curve.

For comparison with the results obtained by the new procedure 24 female rats were di-

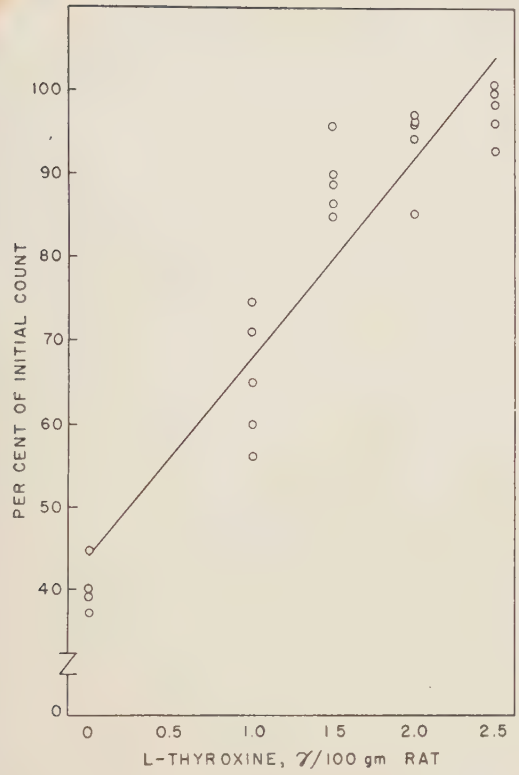

FIG. 3. Effect of progressively increasing thyroxine dosage between groups of rats on I^{131} retention in their thyroids.

vided into 5 groups averaging 273 to 277 g body weight and injected with 40 μc of I^{131}/rat. A base count was taken 120 hours after I^{131} injection. A group of 4 rats received no thyroxine. The other 4 groups, comprising 5 rats each, were injected daily for 6 days with a different level of L-thyroxine given each group, and a final thyroid count was taken on seventh day. The final thyroid counts were expressed as per cent of initial count and plotted against dose (Fig. 3). This is the only instance where we have observed an apparently curvilinear response by this procedure. Because of this, and also for simplicity of treatment, a straight line was fitted to the data. A highly significant correlation of 0.942 between thyroxine dose and per cent of preceding count was found. From regression equation, $y = 44.3 + 23.8x$, the estimated thyroxine secretion was 2.34 μg/100 g body weight.

In agreement with the report of Perry(3) it was found in the foregoing experiments that when an adequate level of thyroxine administration is reached, output of I^{131} from the thyroid is apparently completely inhibited.

As a further check, 21 male rats averaging 200 g body weight were divided into 2 groups comprising 7 and 14 animals and injected with 30 μc of I^{131}/rat. Forty-eight hours later daily L-thyroxine injections were started in both groups at the rate of 1 μg/100 g body weight. Dosage was increased progressively at 2-day intervals, and external thyroid count taken immediately before each increase in dosage. One group (14 rats) was started on 1/10% thiouracil in its drinking water, at the same time the thyroxine injections began. Thyroid counts of the first group again leveled out, as the thyroxine dose reached a critical level, at 99 to 100% of preceding count (Fig. 4). The thiouracil-treated group, however, leveled out at 92 to 93% of preceding count and did not go any higher with increasing thyroxine dose. Thyroid output, based on the last 3 points on the flat part of curve, continued at the rate of 4.2% daily.

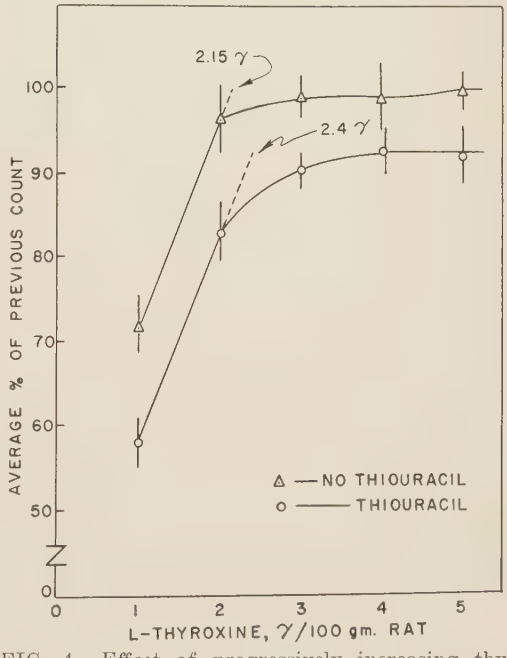

FIG. 4. Effect of progressively increasing thyroxine dosage on avg retention of I^{131} in thyroids of normal as compared to thiouracil treated rats. Vertical bars represent stand. error of mean in each case.

From these results it appears that thyroxine treatment does not completely block I^{131} turnover. In rats that have organic binding of iodine largely blocked by thiouracil administration, I^{131} output continues at a slow rate despite a high dosage with thyroxine. In normal rats at a similar thyroxine dosage this output appears to be counterbalanced by an approximately equal uptake, with the result that there is no net change in thyroid count. The points on the curves where these results are indicated occurred about 8 days after the I^{131} injection when body background counts had reached insignificant levels. The small uptake is probably due, therefore, to recirculation of I^{131} from compounds that have been released from the thyroid and metabolized.

An estimate of thyroid secretion rate in these rats, obtained by extrapolating the ascending response lines to 100% and 92.5% in the normal and thiouracil-treated rats, respectively, gives values of 2.15 and 2.4 μg/100 g body weight. In line with the above discussion, the recycling of I^{131} through the thyroid would be represented approximately by the difference between these values. This amounts to roughly 11% of daily output.

A point of further interest concerns length of time that is required for the thyroid-pituitary balance, as indicated by the thyroid count, to come into equilibrium with a given dose of thyroxine. In a further experiment 22 male rats averaging 249 g body weight were divided into 4 groups and injected with 30 μc of I^{131}/rat. An external thyroid count was taken 72 hours later, and immediately thereafter daily thyroxine injections were started at levels of 0.5, 1.0, 1.5 and 2.0 μg/100 g body weight in the respective groups. Control data were obtained on two additional groups, one receiving I^{131} only and the other receiving 1/10% thiouracil in its daily drinking water subsequent to initial count. As shown by average counts in Fig. 5, I^{131} output by thyroid was altered in all cases within 24 hours of first thyroxine injection. At the 2 μg dosage there was a small accumulation of iodine for the first 3 days. At lower levels the slope of the output curve was decreased in proportion to dosage. Rats receiving thiouracil only had an output ½ time of 1.5 days, compared to 4 days for normal rats. The T½ for groups receiving 0.5, 1.0 and 1.5 μg L-thyroxine/100 g body weight, respectively, was 5.3, 7.3 and 11.2 days.

When individual counts after 4 days of thyroxine dosage expressed as per cent of initial count, were plotted against dosage as in Fig. 3, there was a highly significant correlation of 0.979 between per cent of initial count and thyroxine dose. From regression equation, $y = 43.81 + 25.96x$, the estimated thyroxine secretion rate was estimated as 2.15 μg/100 g body weight.

Discussion. Estimates of thyroid secretion rate obtained by progressively increasing thyroxine dose in individual rats or small groups (Fig. 1, 2 and 4) agree very closely with those obtained where the dose is varied between groups (Fig. 3 and 6). The new procedure has the advantage of permitting estimates of absolute thyroid secretion rate to be made on individuals or small groups of animals.

The observed inhibition of thyroid output by administered thyroxine can be explained by the suppressing effect of thyroxine on thy-

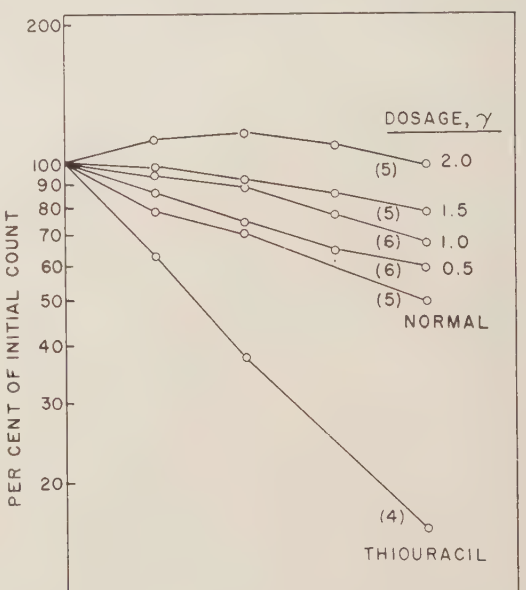

FIG. 5. Effect of varying dosage levels of thyroxine, given continuously to groups of rats on thyroidal I^{131} output curve. Figure in parentheses shows No. of rats included per group.

rotropic hormone output. As shown by Wolff (2) thyroxine administration at high levels suppresses I^{131} output by thyroid to about the same extent as hypophysectomy. Under our laboratory conditions there appears to be a small iodine turnover even in rats receiving thyroxine equivalent to their thyroid secretion rate. In normal rats, output is approximately balanced, however, by the uptake. If organic combination of iodine is blocked by thiouracil, output, even at high thyroxine levels, continues at average rate of 4.2%/day. (Fig. 4). Estimated daily thyroid secretion rate is about 11% higher in thiouracil-treated than in normal rats, because of recycling of I^{131} in the normal rat thyroid. This agrees very well with the estimate of 10.2% daily for recycling of I^{131} by rabbit thyroid that was derived by entirely different methods(6).

The actual thyroid secretion value of 2.15-2.6 μg L-thyroxine/100 g body weight daily obtained under our conditions is somewhat lower than the estimate of 10 μg for rats weighing 170-250 g reported by Perry(3). The most extensive studies by the goitrogen technic(7) yielded values of 2.8-3.5 μg D, L-thyroxine/100 g body weight in adult rats. This is the equivalent of 1.4-1.75 μg L-thyroxine if it is accepted that the L-isomer has twice the potency of the racemic mixture(8).

The principles reported in this paper have been applied successfully to the measurement of thyroid secretion rate of domestic sheep (9). Further research will be required to determine their applicability to other species.

Summary. A procedure is described for determination of the thyroid secretion rate in the intact rat. Based on inhibition of thyroidal I^{131} output by progressively increasing doses of thyroxine, it is applicable to individuals or small groups of animals. Values obtained by progressively increasing thyroxine dosage within individuals compare closely with those obtained by varying dosages between groups. They are also similar to reported values obtained by the goitrogen technic. Thyroid secretion values, expressed as μg L-thyroxine/100 g body weight were 2.21-2.56 for adult female and 2.15 for adult male rats. Rats given thiouracil to prevent further thyroidal combination of I^{131} during treatment yield values about 10% higher than those not receiving thiouracil.

1. Cornwall, R. L., and Reineke, E. P., *Fed. Proc.*, 1951, v10, 30.

2. Wolff, J., *Endocrinology*, 1951, v48, 284.

3. Perry, W. J., *ibid.*, 1951, v48, 643.

4. Albert, A., *ibid.*, 1951, v48, 334.

5. Reineke, E. P., and Turner, C. W., *J. Biol. Chem.*, 1953, v149, 555.

6. Brown-Grant, K., von Euler, C., Harris, G. W., and Reichlin, S., *J. Physiol.*, 1954, v126, 1.

7. Monroe, R. A., and Turner, C. W., *Mo. Agr. Exp. Sta. Res. Bul.* 403, 1946.

8. Reineke, E. P., and Turner, C. W., *Endocrinology*, 1945, v36, 200.

9. Henneman, H. A., Griffin, S. A., and Reineke, E. P., *J. Animal Sci.*, 1952, v11, 794.

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Plasma Half Life of Na^{22} in Dogs. (21538)

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Relatively few studies on sodium metabolism in mammals have been conducted with the radioactive isotope Na^{22} owing to its long physical half life. In connection with an unrelated research we had an opportunity to determine the plasma half life of Na^{22} in a group of pure-bred and inbred beagle dogs. The plasma half life of this isotope appears to be much shorter in dogs kept under usual laboratory conditions than in man.

Materials and methods. Fourteen pure-bred beagle dogs (7 male, 7 female) from an inbred colony were used. All were siblings or half siblings (common sire) 14 months old

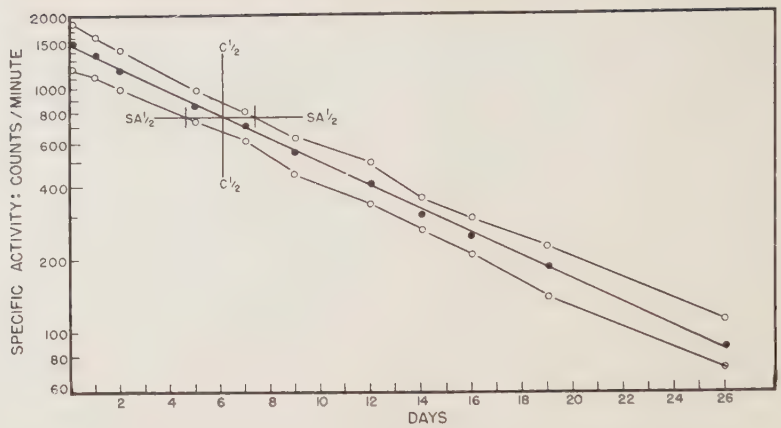

FIG. 1. Disappearance of Na^{22} from plasma of beagle dogs. Solid line—avg of 14 dogs. Broken lines—extremes of variation in counts in each set of samples. $SA\frac{1}{2}$-$SA\frac{1}{2}$ = level equal to ½ of initial plasma specific activity. $C\frac{1}{2}$-$C\frac{1}{2}$ = plasma Na^{22} half life.

(± 0.7 mo.). The dogs were fed measured portions of a diet consisting of Red Heart (commercially canned dog food) and dog meal supplemented with fat which kept their weights constant within 0.1-0.2 kg. The daily sodium intake was 1.52 g. The dogs were fasted overnight and then injected intravenously with 9.2 ml of an isotonic NaCl solution containing a total Na^{22}* activity of 3.59 x 10^6 counts/minute. Blood samples were collected 1, 2, 3, 24, and 48 hours after injection and every 2-3 days thereafter for a total of 26 days. One and one-half ml aliquots of plasma were placed in stainless steel planchets, air dried, and counted with a thin end window Geiger-Muller tube for a minimum of 10,000 counts. Counts were corrected for background, self-absorption, and coincidence whenever necessary.

Results. Fig. 1 shows the average plasma Na^{22} disappearance curve (plotted semilogarithmically) and the extremes of variation encountered. Plasma Na^{22} half life (C-1/2) (1) when calculated from the average curve is 6.1 days with a range of 4.6-7.4 days. If C-1/2 is calculated for each dog individually the average C-1/2 is 6.02 days with a range of 5.0-7.0 days and a standard deviation of ± 0.68.

C-1/2 is not correlated with the weight of the dog, nor with the initial plasma Na^{22} activity as measured by the one hour sample.

Discussion. Threefoot, Burch and Reaser (1) found the average serum half life of Na^{22} to be 13.3 days (range, 12-14 days) in 4 human subjects. Thus, our data would seem to indicate that Na^{22} activity disappears twice as fast from the circulation of the dog as from the human blood stream. A similar observation has been made in the rat(2). Burch, *et al.*(1,3) have shown by theoretic analysis and experiment that the slope of the Na^{22} disappearance curve depends upon the Na intake. Increasing the Na intake increased the slope and decreased C-1/2 while subnormal Na intake decreased the slope and increased C-1/2. Our dogs were maintained on a constant diet which kept them healthy and happy and it must be regarded as fortuitious that the Na intake was such that C-1/2 turned out to be about 6 days. One cannot be certain, however, that this intake represents the optimal Na intake for dogs. The significance of the observed difference between C-1/2 for man and dog depends upon what it considered to be "normal" Na intake for each species. For example, plasma Na^{22} half life in man was 8 days on a high Na diet (13.7 g NaCl/day), but 25 days on a low sodium diet (1.7 g NaCl/day)(1). Our dogs received the equivalent of 3.88 g NaCl/day which is proportionately higher than the human "high"

* The Na^{22} was obtained from Abbott Laboratories on authorization from the AEC.

sodium intake. By restricting Na intake in the dog it appears possible to make the C-1/2 approximate that normally observed in man, but we have no reason for assuming that such a maneuver would result in a more optimal physiological status for the dog. Even so, it is clear that if comparisons of the plasma Na^{22} half life are to be made it will be necessary to do so under conditions of equivalent Na intake.

Na^{22} proved to be most useful for this type of study. No untoward difficulties or hazards requiring other than the usual precautions taken with radioactive material were encountered.

Summary. The average plasma Na^{22} half life in beagle dogs is about 6 days when the average daily Na intake is 1.52 g.

1. Threefoot, S., Burch, G., and Reaser, P., *J. Lab. and Clin. Med.*, 1949, v34, 1.

2. Bauer, G. C. H., *Acta Physiol. Scandinav.*, 1954, v31, 334.

3. Burch, G. E., Threefoot, S. A., and Cronvich, J. A., *J. Lab. and Clin. Med.*, 1949, v34, 14.

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I. Cultivation of Vesicular Exanthema Virus *in vitro* Using Pig Embryo Tissues.*† (21539)

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The necessity for propagation of the virus of vesicular exanthema (VE) in swine has hindered experimentation. This paper describes an inexpensive and convenient method for cultivation of VE virus *in vitro* similar to that employed for virus of foot-and-mouth disease(2).

Materials and methods. Virus. The immunotype B_{51} VE virus was employed because it is the type encountered in states other than California(3,4). *Tissues.* Swine embryos were removed aseptically from uteri of market sows at the slaughterhouse. Within 15 minutes after death, suitable embryos were selected by external palpation of the uterus, the ends of the organ were cut and tied, and placed in a sterile pan. The desired embryo was removed through an incision into the allantoic cavity, to a Petri dish. For the preparation of whole embryo mince, 4 to 5 fetuses 2 to 4 cm in length (3rd to 5th week of gestation) were pooled, cut into pieces about 2 to 5 mm in size, and immersed in 200 ml of Simms-Sanders' "Z" solution(5) containing 1000 units penicillin and 0.65 mg of streptomycin/ml. When fetal skin was desired, embryos ranging from 6 to 10 cm were selected. The skin covering neck and trunk was removed by blunt dissection, minced into pieces approximately 5 mm diameter and stored in Simms-Sanders' "Z" solution as described. The "Z" solution and its contents, while in transit, were kept cold in a portable ice chest but in the laboratory stored at 34°F. Viability and respiration of tissues stored for periods longer than two weeks were found to decrease markedly and were less desirable for virus culture. *Tissue Culture Technics.* Tissue washed free of storage fluids in Simms-Sanders' tissue culture medium was further minced (1 to 2 mm sq), placed into a tube of Simms-Sanders' medium (0.25 g tissue to 5 ml fluid), and held at 37°C to re-establish a normal rate of respiration. For culture about 40 mg of active tissue were transferred to test tubes (25 x 200 mm) containing 14 ml of Baker's fluid(6).

* Project supported in part by grants from Agricultural Research Service, U. S. Dept. of Agriculture, and Public Health Service, National Institute of Health.

† During preparation of this paper, a report upon cultivation and demonstration of cytopathogenicity of VE virus in minced swine embryonic skin, muscle and tongue tissue through 16 serial passages was reported by McClain, Madin and Andriese(1).

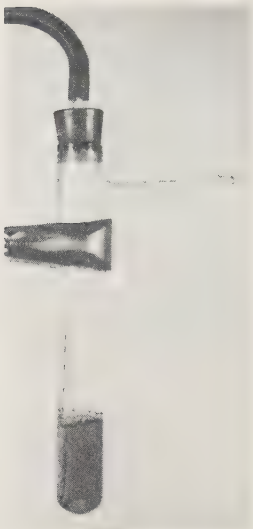

FIG. 1. Method of aerating tube containing Baker's fluid and embryonic tissue for propagating VE virus *in vitro*.

The virus inoculum for the initial culture in each series consisted of 0.25 ml of a clear 10% suspension prepared as described for the preparation of antigen for the complement fixation test(7), except that 5,000 units of penicillin and 30 mg of streptomycin were added during treatment with Lloyd's reagent. During incubation at 37°C for 24 hours, the culture was aerated with carbogen (mixture of 95% O_2 and 5% CO_2) at the rate of 1 ml/min. (Fig. 1). Following incubation, the supernatant fluid was removed, tissues ground in a mortar with alundum, centrifuged at 2500 r.p.m. for 10 minutes, and both supernatant fluids pooled and filtered through a Seitz EK filter. Serial passages were continued with 0.5 ml of the preceding culture. Viability of cultures was determined by inoculating intradermally 1 ml of undiluted culture into snouts of each of 2 susceptible pigs. The animals were observed daily for 12 days and, whenever possible, held in isolation for 3 to 4 weeks before they were challenged with the known virulent parent strain of the virus.

Results. Five separate series of cultures using minced pig embryonic skin suspended in Baker's fluid resulted in successful passage of the virus 4 times once, 5 times twice, 6 times once, and 7 times once. Facilities and cost prohibited titration of each series; however, the extent of vesiculation, incubation period, and severity of the disease in hogs inoculated intradermally into the snout gave some indication of virus content. Cultures serially passed 4 and 5 times produced lesions within 48 to 72 hours, whereas cultures passed 6 and 7 times resulted in a prolonged incubation period of 4 to 6 days before primary lesions were evident, followed by secondary lesions 24 hours later. Although this method is neither valid for determining virus concentration nor indisputable proof of propagation, nevertheless, the same type strain and volume of virus which was used in the inoculum was shown in preliminary experiments to be incapable of surviving in carbogenated Baker's fluid at 37°C for more than 96 hours. Demonstration of the virus in cultures, passed beyond the fifth time, suggested minimal multiplication or support of survival of the virus. Modification of the technic using Simms-Sanders' medium and incubating cultures for 24 or 48 hours by procedures employed for propagation of hog cholera and Newcastle disease virus(8,9), resulted in innocuous cultures following 6 and 7 serial passes, respectively.

Minced whole pig embryos substituted for embryonic skin were found to be far more satisfactory. The results, Table I, showed that the virus could be cultivated and, thus far, has been passed 113 times without show-

TABLE I. Reaction of Swine to Inoculation with Virus Passed in Baker's Fluid Containing Minced Embryos of Swine.

Serial passage	Days tissues stored	Lesions in swine inoc. IDSn* Primary (hr)	Secondary	Challenge†
7	9	24	0	im‡
11	5	48	0	—
15	9	24	0	im
18	2	48	96 hr	"
23	8	48	120 hr	"
28	13	48	0	"
33	1	48	0	"
55	7	96	9th day	—
68	7	72	0	—
99	5	72	96 hr	im
113	7	48	96 hr	"

* IDSn = Intradermally into snout.
† Inoculated IDSn with 1 cc of 10% suspension of type B_{51} virus 3 to 4 wk following exposure.
‡ im = Immune.

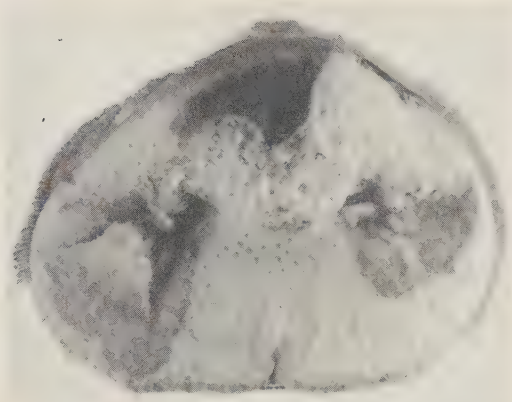

FIG. 2. Typical primary ruptured vesicles on snout of hog inoculated 36 hr previously with culture propagated *in vitro*.

ing any readily recognizable changes in its behavior when inoculated intradermally into snouts of susceptible hogs. The agent produced typical vesicular exanthema in swine, including fever followed by primary vesiculation at points of inoculation (Fig. 2) with or without secondary lesions on hooves and development of immunity to parent strain of the virus. Two swine hyperimmunized to type B_{51} VE were refractory to an intradermal inoculation with the 113th serial culture. To determine the virus concentration, one hundred-fold dilutions of the 28th serial passage which had been stored for 126 days in the dry ice chest were each inoculated intradermally into snouts of two pigs, 4 months old. Excellent lesions were found in hogs inoculated with undiluted and with the 10^{-2} dilution. One of the pigs inoculated with the 10^{-4} dilution presented primary vesicles. All swine showing vesicles were immune to a challenge dose of virus 15 days later. These results indicate that the virus can be propagated in tissue culture to relatively high infectivity titers. Infective cultures of the virus in 250 and 500 ml quantities have been prepared with the method described.

Summary and conclusions. A rapid, simple, and inexpensive method of propagating vesicular exanthema virus of swine was accomplished in a medium of Baker's fluid containing minced swine embryos harvested from sows in the third to the fifth week of gestation.

Grateful acknowledgement is made to Dr. Jacob Stong, inspector-in-charge of the packing plant, for his efforts and patience in supplying the swine embryos, and to Messrs. R. Ryamond and E. Stuart for their valuable technical assistance.

1. McClain, M. E., Madin, S. H., Andriese, P. C., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 771.

2. Frenkel, H. S., *Am. J. Vet. Res.*, 1950, v11, 371.

3. Mott, L. O., personal communication.

4. Bankowski, R. A., *Am. J. Public Health,* 1954, v44, 1119.

5. Simms, H. S., and Sanders, M., *Arch. Path.*, 1942, v33, 619.

6. Parker, R. C., *Methods of tissue culture.* Paul B. Hoeber, Inc., New York, 1938, p76.

7. Bankowski, R. A., Wichmann, R., and Kummer, M., *A. J. Vet. Res.*, 1953, v14, 145.

8. Boynton, W. H., *Vet. Med.*, 1946, v41, 346.

9. Bankowski, R. A., and Boynton, W. H., *Vet. Med.*, 1948, v43, 305.

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Antihemophilic Factor (AHF) Levels Following Transfusions of Blood, Plasma and Plasma Fractions.* (21540)

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It has been recognized for many years that normal plasma has a corrective influence on the clotting defect in hemophilia. The prolonged clotting time is shortened, and the impaired consumption of prothrombin in clotting blood is rectified(1). The effect of normal plasma is attributed to a plasma protein, antihemophilic factor (AHF), which is deficient in hemophilic blood. The present investigation was undertaken (a) to determine the extent to which injected AHF persists in the circulation of normal and hemophilic subjects, and (b) to study the influence of various levels of plasma AHF on the results obtained in clotting time, prothrombin utilization and partial thromboplastin time tests(2). A preliminary report of this work was made recently(3).

Materials and methods. Two human and 11 canine hemophiliacs(4) and 2 normal dogs were used as subjects in these experiments. The human hemophiliacs had been hospitalized because of bleeding. The canine hemophiliacs were in a quiescent phase, free of hemorrhage, and in most cases had not been transfused for at least a month prior to use in these experiments. Blood for study was obtained by the two-syringe method from the antecubital veins of humans and the external jugular veins of dogs. Blood for human transfusions was collected from normal donors into vacuum bottles (Cutter Labs) containing ACD solution (1.32% sodium citrate, 0.48% citric acid, and 1.47% dextrose) in the ratio of one part to 4 parts blood. Plasma was freshly prepared, without freezing or pooling. Blood for canine transfusions was collected from normal kennel dogs, and was mixed immediately with 0.11 M sodium citrate solution in a ratio of one part to 8 parts blood. AHF fractions were prepared from dog plasma by precipitation with ethanol after adsorption with $BaSO_4$ and fuller's earth(5). The product was made up in normal saline buffered with phosphate, pH 7.2. On a volume basis, AHF in the fractions was 2.95-7.5 times as concentrated as in normal plasma. On the basis of nitrogen determinations the fractions were 16-79 times purified. Immediately prior to transfusion of the dogs, a volume of blood approximately equal to that being administered was withdrawn in an attempt to minimize hypervolemia. Blood and plasma were injected at the rate of 10 ml per min, plasma fractions at a somewhat slower rate. Four separate clotting tests were done at intervals to study the effects of transfusion: (a) whole blood clotting time, 3-tube (10 x 75 mm) method; (b) prothrombin utilization in whole blood 30 min after collection, using a modified two-stage procedure(6); (c) partial thromboplastin time (PTT) with 0.3% crude cephalin(2); and (d) AHF assay(2) to determine plasma AHF levels as % of normal homologous plasma.

Results. Transfusions to hemophilic subjects. AHF was given as a single transfusion

TABLE I. Normal Plasma Transfusion to a Hemophilic Dog: Effect on Clotting Tests.*

Time after transfusion (hr)	Clotting time (min.)	Prothrombin utilized in 30 min. (%)	PTT (sec.)	AHF (%)
0	120	4	180	0
½	9	87	60	22
1	8½	90	57	21
2	9	90	64	16
4	10½	90	72	12
6	10½	87	158	tr†
24	10½	89	167	"
48	18	67	175	"
96	45	19	162	"
144	60	8	197	"

* 13 kg male hemophilic dog given 23 ml normal plasma/kg body wt.

† tr = Values below 5% for the AHF assay are recorded as a trace.

* This investigation was supported in part by research grant from the National Heart Institute, National Institutes of Health, Public Health Service.

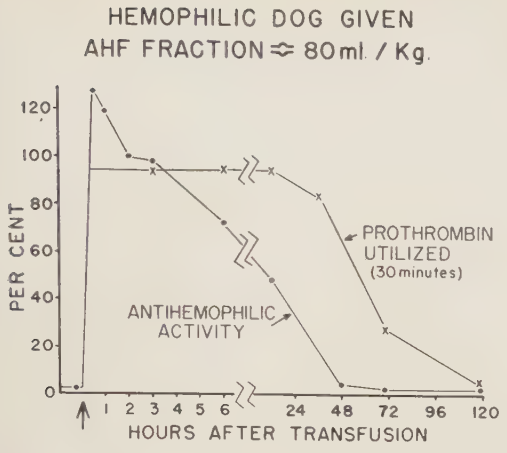

FIG. 1. Antihemophilic activity and prothrombin utilization tests in a 9.7 kg hemophilic female dog after transfusion with a plasma fraction. 103 ml of a plasma fraction, 7.5× as concentrated as normal plasma, 79× purified with respect to protein nitrogen, injected to give a total dose equivalent to 80 ml normal plasma per kg. Start of infusion indicated by arrow. Total injection time 12 min.

of normal homologous blood or plasma in 12 separate experiments, 10 with dogs and 2 with humans. In each experiment, the highest level of AHF was found immediately after transfusion. Thereafter it fell rapidly. Only traces were detectable after 3-4 hours. A typical experiment is shown in Table I. Although only traces of AHF were present 6 hours after transfusion, clotting times and prothrombin utilization were within normal limits for 24 hours. By contrast, PTT was in the normal range for only the first 2 hours, during which time AHF levels fell from 22 to 16%. At lower AHF levels the PTT was prolonged. In these 12 experiments, the volumes of the transfusions varied as follows: group A, 4 dogs given whole blood, 12.5-21 ml per kg body weight; group B, 2 humans given whole blood, 3.1-8.2 ml per kg; and group C, 6 dogs given plasma, 14-23 ml per kg. The maximum AHF level in each of the groups was observed in the subject receiving the largest dose; these values for the 3 groups were 19, 7 and 23% respectively. To attain *higher levels* of AHF, a single injection of a plasma fraction rich in AHF was given to 2 hemophilic dogs. AHF was increased to supernormal levels temporarily. The results of one experiment are shown in Fig. 1. The AHF remained at a high level for over 18 hours. The divergent results obtained with the prothrombin utilization test are again seen—rapid utilization was still observed 36 hours after transfusion.

In an attempt to maintain AHF at elevated levels over a long period of time, repeated plasma transfusions were given to 2 dogs and one patient. In the dogs, AHF was maintained well above the 5% level only by giving adequate volumes of plasma, about 7 ml per kg, at relatively short intervals of 2-4 hours. With such frequent transfusions, an additive effect on AHF was observed with each succeeding dose. This principle of frequent transfusions was used successfully in the therapy of a patient bleeding subcutaneously in the scalp. Plasma in the amount of 3.7 ml per kg was given every 4 hours for 24 hours. AHF levels of 6-14% were maintained and bleeding ceased. During the next 24 hours, plasma in the same amount was given but at longer intervals, every 8 hours. AHF rose to about 5% after each transfusion, but only traces could be detected at the end of each 8 hour period.

Transfusions to normal subjects. Two normal dogs were given an AHF-rich plasma fraction. AHF levels were increased to more than twice the normal level. The results of

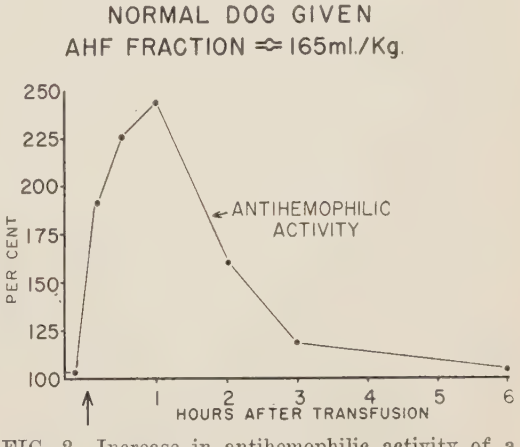

FIG. 2. Increase in antihemophilic activity of a 4.6 kg normal male dog after transfusion with a plasma fraction. 103 ml of a plasma fraction, 7.3× as concentrated as normal plasma, 53× purified with respect to protein nitrogen, injected to give a total dose equivalent to 165 ml normal plasma per kg. Start of infusion indicated by arrow. Total injection time 30 min.

TABLE II. Results of Clotting Tests in Mixtures of Normal and Hemophilic Dog Bloods.*

(1) Normal-hemophilic blood mixture (% normal)	(2) AHF (%)	(3) PTT (sec.)	(4) Clotting time (min.)	(5) Prothrombin utilized in 30 min. (%)
100	100	68	7	96
50	54	67	7	90
25	20	70	7	90
12.5	15	82	7	90
6.25	8	99	7	90
3.12	tr†	110	7	90
1.56	"	130	7	90
0.77	"	170	12	50
0.0	0	280	60	8

* Hct values: normal 42%; hemophilic 41.5%.
† tr = trace.

one of these experiments are shown in Fig. 2. The high level of AHF was maintained only briefly. Half of the increase was lost in less than 2 hours, and the normal level was re-established in about 6 hours.

Relation of results of clotting tests to AHF levels. The preceding *in vivo* experiments suggested that the various clotting tests used were sensitive indicators of plasma AHF within only a limited range of this clotting factor. Results of an experiment designed to study these relationships further are shown in Table II. Freshly drawn normal and hemophilic canine bloods were mixed immediately in varying ratios to give a broad range of AHF values. It is seen that the expected and actual results for AHF (columns 1 and 2) agree very well. The PTT was increasingly prolonged as the AHF levels were decreased below 15%; higher levels caused little or no change. Clotting time and prothrombin utilization tests gave normal values except with AHF levels of less than 1%; these two tests did not appear to be influenced by changes in AHF above this level. Comparable experiments with mixtures of normal and hemophilic human bloods gave similar results.

Discussion. In hemophilia the increase in plasma AHF levels following transfusion is dependent upon the amount of AHF administered. Because of the volumes required, it is difficult to attain a level of more than 20-25% of normal with whole blood or plasma transfusions. High levels of AHF, even exceeding normal, may be obtained by administration of plasma fractions rich in this clotting factor. Similar observations have been made by Macfarlane, Biggs and Bidwell(7), using a bovine AHF fraction in patients. Half the initial increase in AHF activity was maintained only 2-3 hours following injection of whole blood or plasma. Even by injection of large amounts of concentrated AHF significant levels were maintained for only about 18 hours. Amounts of AHF sufficient to influence whole blood clotting time and prothrombin utilization studies remain in the circulation for several days. Actually this represents only trace amounts.

Two important considerations in transfusion therapy for hemophilic bleeding are attainment of the minimum level of AHF required for hemostasis and maintenance of this level. The minimum hemostatic level of AHF has been postulated to be not greater than 20% of normal(8). The prompt control of hemorrhage in the patient in whom the AHF was maintained between 6 and 14% suggests that AHF requirements may be even lower. Hemorrhages in hemophilic patients are often controlled with an amount of plasma which probably would not raise the AHF level above 5%. Regardless of what the exact hemostatic level of AHF may be, frequent replenishment of this factor appears essential because of its rapid utilization. From the data presented here, it would appear necessary to administer AHF in the form of plasma or whole blood every 2-4 hours if AHF levels even in the range of 5-10% are to be maintained.

One hypothesis of the nature of hemophilia is that AHF is produced in normal amounts, but is not available for participation in clotting. It is postulated that AHF may form an inactive complex with an inhibitor, or that it may be lost from the circulation by rapid filtration into the tissues or urine. If such mechanisms are responsible for the hemophilic state, one would expect that the activity of injected AHF would be maintained in the circulation of normal subjects for a much longer time than in hemophilic subjects. Such was not the case. In fact, AHF given to normal dogs disappeared more rapidly than that

given to hemophilic animals (*cf* Fig. 2 and 3).

Recent *in vitro* studies(9) have shown that serum accelerator factors (SAF) may influence clotting time and prothrombin utilization tests done on hemophilic blood. Traces of SAF might have been activated in the blood or plasma used for transfusions. It is unlikely that such traces would be an influence in our *in vivo* experiments, since Graham(10) has injected a potent SAF preparation (150 units per ml, 23 units per kg) into hemophilic dogs without causing any change in either whole blood clotting time or prothrombin utilization.

It has commonly been assumed that the laboratory diagnosis of hemophilia, even of severe forms, is impossible during active transfusion therapy. This is undoubtedly true if one relies only on clotting time or prothrombin consumption tests (Table II). The PTT test will demonstrate the existence of a clotting defect unless AHF has been raised to levels above 15%. In any event, actual determination of AHF will reveal the hemophilic state.

Summary. 1. Canine and human hemophilic subjects were transfused with homologous blood or plasma. Dogs were also transfused with AHF-rich plasma fractions. The increase in plasma AHF levels following transfusions was proportional to the amount of AHF administered. Over half of the AHF activity disappeared within a few hours, although traces of AHF may persist for nearly a week. Frequent replacement transfusions are required if AHF levels as high as 5-10% are to be maintained. 2. Injection of potent plasma fractions into normal dogs resulted in transient supernormal AHF levels. Half of the injected AHF was lost in about 2 hours. 3. The minimum amount of AHF required to give normal values is not uniform with different clotting tests. AHF levels above 1% resulted in normal values for the clotting time and prothrombin utilization tests. Levels above 15% resulted in normal values for the PTT test.

1. Brinkhous, K. M., *Am. J. Med. Sci.,* 1939, v198, 509.

2. Langdell, R. D., Wagner, R. H., and Brinkhous, K. M., *J. Lab. Clin. Med.,* 1953, v41, 637.

3. ———, *Fed. Proc.,* 1954, v13, 436.

4. Graham, J. B., Buckwalter, J. A., Hartley, L. J., and Brinkhous, K. M., *J. Exp. Med.,* 1949, v90, 97.

5. Wagner, R. H., Pate, D., and Brinkhous, K. M., *Fed. Proc.,* 1954, v13, 445; Wagner, R. H., unpublished data.

6. Langdell, R. D., Graham, J. B., and Brinkhous, K. M., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 424.

7. Macfarlane, R. G., Biggs, R., and Bidwell, E., *Lancet,* 1954, v266, 1316.

8. Graham, J. B., McLendon, W. W., and Brinkhous, K. M., *Am. J. Med. Sci.,* 1953, v225, 46.

9. Graham, J. B., Langdell, R. D., Morrison, F. C., Jr., and Brinkhous, K. M., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 45.

10. Graham, J. B., unpublished data.

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Effect of Chlortetracycline on Chick Thyroid Size. (21541)

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Calesnik, Harris, and Jones(1) have reported some evidence to suggest that chlortetracycline and penicillin have a goitrogenic and antithyroid effect in young rats. This action was demonstrated by an increased thyroid weight and a decreased uptake of I^{131}. However, Libby and Meites(2), in a single experiment, were unable to substantiate these findings even though greater quantities of the antibiotics were fed to rats. These latter workers were also unable to demonstrate a goitrogenic effect of penicillin in chicks. Recently, Mellen and Waller(3) reported data to show that an aureomycin concentrate, when fed to chicks, increased the size of the thyroid gland significantly. The results presented in the present paper are from data obtained from four separate replications covering a period of 6 months extending from April through September, 1954.

Procedure. In each of the 4 experiments, day-old chicks were distributed into uniform lots of 10 each on the basis of body weight. New Hampshire female chicks were used in Exp. 1 and 4*; crossbred male chicks† in Exp. 2 and 3. The basal diet (Table I) was used in all of the trials and was supplemented, as shown in Table II, with different levels of chlortetracycline. A group of chicks receiving the basal diet supplemented with 0.2% thiouracil was also included in each of the 4 trials. The chicks were maintained in electrically heated batteries, and feed and water were supplied *ad libitum*. At the end of 4 weeks, the birds were weighed, sacrificed, and the thyroids were removed and weighed individually on a Roller-Smith balance. The body cavity of each bird was opened at time of sacrifice and the sex noted.

Results. The data are summarized in Tables II and III. A highly significant increase in both the actúal thyroid weight and the thyroid/total body weight ratio was observed in Exp. 1 in the groups receiving 50 and 100 mg of chlortetracycline per kilo of diet, and in Exp. 2 in those groups receiving 100 and 1000 mg of chlortetracycline per kilo. In Exp. 3, a significant increase in only the actual thyroid weight was noted. However, the thyroid/total body weight ratio for the group receiving 1000 mg of the antibiotic per kilo of diet was significant at the 10% level. In Exp. 4, a slight but non-significant increase in actual thyroid weight was observed in those groups receiving chlortetracycline. However, examination of the data obtained from the first 3 replications by analysis of variance, shows a highly significant treatment effect (Table III).

TABLE I. Composition of Basal Diet.

	%
Ground yellow corn	59.35
Soybean meal	26.
Alfalfa leaf meal	5.
Fish meal	6.
Steamed bone meal	1.
Limestone flour	1.
Butyl fermentation solubles (500 μg riboflavin/g)	.6
Salt mixture (+4% $MnSO_4$ $4H_2O$)	.5
Methionine	.2
Vit. A and D oil (2250A, 600D)	.1
Choline supplement (98,415 mg/lb)	.15
Vit. B_{12} concentrate (6 mg/lb)	.1
	mg/kg
Folic acid	2.
Calcium pantothenate	3.

Protein (calculated) 21.6%.

Examination of the actual thyroid weight from the 4 replications by regression analysis gave b-values of 0.482, 0.046, 0.043, and 0.037. The b-values for the thyroid/total body weight ratios were 0.947, 0.011, 0.012, and 0.011. Thus the thyroid response is closely correlated to increased antibiotic dosage in only the first replication. Progressively increasing antibiotic dosage above the 100 mg/kilo level did not increase thyroid

* 80% of chicks in Exp. 4 were females.

† New Hampshire male x Silver Cornish female.

TABLE II. Effect of Chlortetracycline on Chick Thyroid Size.

	mg/kg	Avg body wt 4 wk (g)	Avg* thyroid wt (mg) Actual	Avg* thyroid wt (mg) Per 100 g B.W.
Exp. 1 (April)				
Control		359	24 ± 2	6.7 ± .5
Chlortetracycline	10	354	25 ± 3	7.3 ± .6
"	50	340	32 ± 3†	9.3 ± .7
"	100	374	42 ± 2‡	11.4 ± .5‡
Thiouracil 0.2%		273	355 ± 68‡	141.4 ± 19.9‡
Exp. 2 (May)				
Control		379	24 ± 2	6.3 ± .5
Chlortetracycline	10	401	24 ± 2	5.9 ± .5
"	100	444	34 ± 2‡	7.7 ± .4
"	500	449	35 ± 2‡	7.6 ± .4
"	1000	423	37 ± 3‡	8.6 ± .6‡
Thiouracil 0.2%		231	164 ± 33‡	71.1 ± 14.1‡
Exp. 3 (June)				
Control		324	25 ± 2	7.8 ± .9
Chlortetracycline	100	372	30 ± 1†	8.0 ± .1
"	500	371	25 ± 2	8.0 ± .5
"	1000	369	37 ± 2‡	9.8 ± .6
Thiouracil 0.2%		206	145 ± 26‡	60.3 ± 10.4‡
Exp. 4 (Sept.)				
Control		320	26 ± 2	8.1 ± .4
Chlortetracycline	100	337	26 ± 2	7.9 ± .8
"	500	350	28 ± 1	8.1 ± .3
"	1000	328	30 ± 1	9.1 ± .5
Thiouracil 0.2%		142	188 ± 22‡	76.3 ± 10.9‡

* Avg and stand. error.
† Significantly different from control group at 5% level (t-test).
‡ " " " " " 1% " " .

size at the same rate as that observed at the lower levels.

Since these trials covered a period of 6 months and the effect of chlortetracycline on thyroid size was less in the later replications, a seasonal response to chlortetracycline by the thyroid is suggested. It was noted that the maximum growth response to chlortetracycline was obtained when the antibiotic was fed at a level of 100 mg/kilo of diet. As stated above, a similar effect on thyroid size was also noted. The thyroidal enlargement produced by feeding 0.2% thiouracil was much greater than that observed in the groups receiving chlortetracycline. The thiouracil treatment also produced the expected growth depression.

TABLE III. Analyses of Variance of Actual Thyroid Weight.

Exp.	Source of variation	d.f.	m.s.	F-ratio
1	Control versus drug	1	657.02	11.92*
	Levels of drug	2	680.49	12.34*
	Error	34	55.13	
2	Control versus drug	1	551.91	13.92*
	Levels of drug	3	352.07	8.88*
	Error	44	39.65	
3	Control versus drug	1	292.07	10.07*
	Levels of drug	2	119.51	4.12
	Error	34	29.01	
4	Control versus drug	1	39.59	1.79
	Levels of drug	2	60.65	2.74
	Error	27	22.17	

* Significant at 1% level.
Data from birds receiving thiouracil not included in table.

No attempt is made to explain the mechanism of the action of chlortetracycline on the thyroid gland based on these data. Chlortetracycline caused an increased thyroid size without a concurrent reduction in growth rate. Indeed, in most cases, growth was stimulated by this treatment. Apparently chlortetracycline did not act in the same manner as does thiouracil which inhibits thyroxine formation and thus interferes with growth. These data permit the postulation that the antibiotic stimulated the pituitary to increase its output

of thyrotropic hormone, which, in turn, increased the size and possibly the activity of the thyroid gland.

Summary. Chlortetracycline was fed to chicks at levels of 10, 50, 500, and 1000 mg/kilo of diet. The data show that this treatment significantly increased the size of the thyroid gland. Examination of the data by regression analysis gave b-values to show that antibiotic dosage up to 100 mg/kilo of diet was correlated to increase in thyroid size. Increasing the antibiotic dosage above this level did not increase the thyroid size at the same rate. Since the growth of the chicks was not depressed and the thyroid was not enlarged as much as it was when thiouracil was fed, it appears that chlortetracycline did not duplicate the action of thiouracil.

1. Calesnick, B., Harris, W. D., and Jones, R. S., *Science,* 1954, v119, 128.

2. Libby, D. A., and Meites, J., *ibid.,* 1954, v120, 354.

3. Mellen, W. J., and Waller, E. F., *Poultry Sci.,* 1954, v33, 1036.

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Fluid Draining from Functionally Distended Kidney.* (21542)

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When a hemostat is set on the renal pedicle, thus occluding the kidney's artery, vein and ureter, and then the kidney removed and the hemostat unclamped, a considerable quantity of fluid flows out. Its volume is equal to 22% of the volume of the normally distended kidney(1). Two aspects of this phenomenon have been investigated: the source of the fluid and its composition.

Methods. Dogs were used, anesthetized with sodium pentobarbital. The kidney was isolated by a flank approach, the dog being suspended on hooks in the standing position so that the kidney could be well visualized. All collateral circulation to and from the capsule was obviated by dissecting the kidney free from the peritoneum and other membranes, leaving the organ finally isolated from the body except for the artery, vein and ureter. After the surgical procedures, some 30 minutes were allowed to pass in order to permit recovery from trauma. 1. *Source of draining fluid.* After putting the hemostat on the vessels of the pedicle, either the artery or the vein was cannulated and the quantity of fluid draining was measured. It was found that almost the entire volume of 5-15 ml, depending directly on the size of the kidney, drained from the vein, whereas only 0.1-0.5 ml drained from the artery. None, furthermore, drains from the ureter: before clamping the hilar vessels, it was catheterized with polythene tubing, the tip of the catheter being run into the pelvis. Then the artery and vein were simultaneously clamped. Urine flow ceased at once; there was no spurt of urine from the catheter, not even of a few drops. But when the vein was cut, the entire volume of fluid drained out, amounting, as indicated above, to about 22% of the functionally distended organ. 2. *Composition of fluid.* The ureter was catheterized in all experiments to make sure that the kidneys were functioning normally. An arterial blood sample was then drawn from the carotid artery, using heparin as anticoagulant. A few seconds later the renal blood vessels were doubly clamped, the kidney removed by cutting between the clamps and the organ put into a beaker. The vein was quickly cut and the organ allowed to bleed freely, heparin being used as anticoagulant. The kidney was not touched or

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TABLE I. Analyses of Arterial Blood and Kidney Samples.

Dog. No.	Kidney No.	Sample from:	Hemato-crit, %	Plasma protein, g %	Na, mE/L	K, mE/L	Cl, mE/L	Vol of fluid draining†
I	A	A*	31	7.1	151	4.7	114	
		K	12	6.4	151	5.5	145	24
	B	A	32	6.2	147	4.3	109	
		K	15	3.4	143	7.9	125	22
II	A	A	50	5.4	147	6.4	116	
		K	34	4.4	153	14.5	123	13
	B	A	48	4.0	134	5.5	108	
		K	30	2.9	138	10.5	127	30
III	A	A	43	4.7	140	4.7	142	
		K	30	3.8	140	7.7	132	13
	B	A	40	3.4	140	5.2	100	
		K	32	2.9	120	7.7	124	21
IV	A	A	44	4.7	120	4.9	114	
		K	34	2.9	115	10.3	130	19
	B	A	49	4.9	115	4.8	110	
		K	34	2.2	120	10.5	126	17

* A = Artery; K = Kidney.

† Expressed as ratio of volume of fluid draining to volume of distended kidney times 100, *i.e.* % of functionally distended kidney.

squeezed, but the beaker was gently whirled in order to mix the escaping fluid with anticoagulant. About five minutes were allowed for the process of drainage. (We shall designate the samples collected in this manner as "kidney fluid.") The following analyses were done on all samples: a. Hematocrit. b. Plasma proteins by the Biuret method(2). c. and d. Plasma sodium and potassium with a Beckman spectrophotometer with flame attachment. e. Plasma chloride by the method of Schales and Schales(3).

Table I shows the *results*. Each of the 4 dogs contributed 2 kidneys to the experiment. In all cases, the vein of the first kidney (No. A) was opened 30 seconds after the renal artery and vein had been clamped but the vein of the second kidney (No. B) was opened 2 minutes after the 2 vessels had been clamped. The experiment was done in order to ascertain whether a short delay in opening the vein would influence the results. It will be appreciated from inspecting the table that such a delay had no clear-cut effect.

The fluid draining from the kidneys averaged 20% of their functionally distended volumes. It differed from arterial blood in many respects. There is considerable individual variation, as Table I shows, but the trend in all cases is uniform: the kidney fluid has a relatively low hematocrit. Its plasma has less protein than the arterial sample, the same quantity of Na, and more K and Cl. In Table II are shown the averages of these analyses and also the ratios of the content of the substance in the kidney sample to that in arterial blood.

The fluid draining is evidently a mixture of blood with some other fluid; we shall refer to this additional fluid as "diluting fluid." Many of its characteristics may be calculated. Taking the hematocrit as index of dilution, the volume of the "diluting fluid" (V) is given by the formula: $V = \frac{H_A - H_K}{H_K}$ when H_A and H_K refer to the hematocrits of arterial blood and kidney fluid, respectively. The volume is found to average 0.5 ml of diluting fluid added to each ml of blood. The fluid draining from the kidney under the conditions of the experiment is therefore about 1 part diluting fluid to 2 parts whole blood, or some 6.7 ml of diluting fluid and 13.3 ml of blood per 100 ml of functionally distended kidney.

The composition of the diluting fluid may also be calculated with respect to protein and

TABLE II. Averages and Ratios of Substances in Arterial Blood, Kidney Fluid, and Diluent of Kidney Fluid.

	Hematocrit	Plasma protein	Na	K	Cl
S_A*	42	5.1	137	5.1	114
S_K	28	3.6	135	9.3	129
S_K/S_A	.67	.71	1.0	1.8	1.1
S_D		1.9	133	14.2	146
S_D/S_A		.35	1.0	2.8	1.3

* S refers to quantity of given substance, in units used in Table I. Subscripts A, K and D refer to arterial blood, kidney fluid and diluent of kidney fluid, respectively.

salts. The following formula was used:†

$$S_D = S_K - \frac{100 - H_A}{100\,V}(S_A - S_K)$$

when S_D, S_K and S_A refer to the substance whose concentration is being examined, whether for diluting fluid, kidney fluid or arterial blood respectively. It is assumed in the calculation that the hematocrit gives an accurate measure of the volume of the diluting fluid. It is also assumed that the formation of urine exerts a vanishingly small influence on the blood passing through the kidney, an assumption felt justified because blood flow through the kidney is so large that arterio-venous differences for the substances under consideration are probably insignificant (see 4,5).

In Table II are shown the results of the calculations, the amounts of the various substances in the diluting fluid and their ratios to arterial blood content being shown. The diluting fluid contains, apparently, 1.8 g % protein, over 2 times more K than blood plasma, 1.3 times more Cl than blood plasma and the same quantity of Na.

Discussion. The origin of the fluid draining from the kidney, under the conditions of the experiment, is enigmatic. There is reason to believe that it is none of the following: 1) blood from which urine has been abstracted, 2) a mixture of blood and interstitial fluid, 3) a mixture of blood and lymph, or 4) a mixture of blood and intracellular fluid. First, if it were blood from which urine had been abstracted, it should contain less water than blood. But it contains more, using either the hematocrit or the plasma proteins as index of hemodilution. Second, it is presumably not a mixture of blood and interstitial fluid, since both fluids contain about the same quantity of K and Cl(6), whereas the kidney fluid is high in the two ions. Third, the same course of reasoning indicates that it is not a mixture of blood and lymph(7). Fourth, because intracellular fluid has little or no NaCl in it(6), if it were mixed with blood, the resultant fluid would be low in NaCl. But the kidney fluid is normal in Na content and high in Cl. In summary then, the kidney fluid appears to be none of the four special fluids mentioned.

There remains the possibility that it is a mixture of blood and tubular urine. It is our present hypothesis that this is the case and that the kidney is functionally distended with urine as well as blood. The tubular urine would perhaps start to reenter the vascular system at once after the artery and vein had been clamped; the osmotic pressure of the plasma proteins would act to draw tubular water, along with small-particle solutes, back into the vascular system. Then when the vein was cut, the mixture of fluids would drain out. If the hypothesis is correct, the kidney fluid and, *pari passu,* the diluting fluid should reflect the tubular composition of various substances. Thus, the high content of Cl is perhaps a reflection of the high level of Cl known to be present in proximal tubular urine(8). Similarly, the composition of pelvic urine is a partial reflection of the composition of tubular urine; hence, if our hypothesis is correct, pelvic urine should be related in some manner to the fluid draining from the functionally distended kidney. These data are now being obtained. Until they become available, further speculation about the source and nature of the fluid is idle.

Summary. When the renal artery and vein and ureter are simultaneously occluded and then the renal vein cut, there flows out of the kidney a volume of fluid equal to about 20% of the functionally distended kidney. Little or no fluid flows out, in the same experiment, if only the artery or the ureter is cut. The fluid draining from the vein was analyzed,

† The derivation of this formula will be given in a subsequent report.

along with a simultaneously drawn sample of systemic arterial blood. The ratio of the kidney fluid to the arterial blood for hematocrits was 0.67; those for the 2 plasmas were: for plasma proteins, .71; for Na, 1.0; for K, 1.8; and for Cl, 1.1.

1. Swann, H. G., in *Renal Function,* Trans. of 3rd Conference, Josiah Macy, Jr. Foundation, p 89, 1952.

2. Weichelsbaum, T. C., *Am. J. Clin. Path.,* Tech. Suppl., 1946, v10, 40.

3. Schales, O., and Schales, S. S., *J. Biol. Chem,* 1941, v140, 879.

4. Van Slyke, D. D., Rhoads, C. P., Hiller, A., and Alving, A. S., *Am. J. Physiol.,* 1934, v109, 336.

5. Mason, M., Blalock, A., and Harrison, T., *ibid.,* 1937, v118, 667.

6. Gamble, J. L., *Chemical Anatomy, Physiology, and Pathology of Extracellular Fluid,* Harvard University Press, Cambridge, Mass., 5th Ed., 1952.

7. Drinker, C. K., and Field, M. E., *Lymphatics, Lymph, and Tissue Fluid,* Williams & Wilkins Co., Baltimore, 1933.

8. Walker, A. M., Bott, P. A., Oliver, J., and MacDowell, M. C., *Am. J. Physiol.,* 1941, v134, 580.

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A Practical Method of Anesthesia for Prolonged Surgical Procedures on the Goat.* (21543)

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Anesthetic procedures may be associated with a high mortality in the goat when the operating time exceeds one hour. A progressive anoxia results from the accumulation of secretions in the very long trachea of the goat. These secretions are primarily derived from continued and excessive salivary secretion that is not blocked by atropine in doses of 0.5 to 1.0 mg. The short bite and long trachea of the goat make intubation and tracheal aspiration by the oral route practically impossible. Along with anoxia, the depression of respirations caused by intravenous sodium pentobarbital anesthesia becomes apparent as the operation continues for a considerable period of time. Ether anesthesia has also been used and difficulties similar to those with sodium pentobarbital have been observed.

Uniformly good results were obtained when a low tracheotomy was combined with intravenous anesthesia that was administered by small, repeated injections of sodium pentobarbital into a continuous intravenous jugular vein drip. The head of the goat was always placed in a dependent position over the edge of the operating table so that salivary secretions could drip freely from the nose and mouth. Tracheal compression resulting from this position caused no difficulty because of the free airway achieved with the low tracheotomy.

Lengthy (4 to 6 hr) surgical procedures were carried out in a series of 10 goats, using this anesthetic method. All goats tolerated the anesthesia without evidence of anoxia or other complications related to the anesthetic method. The tracheotomy tubes usually were removed on the fourth postoperative day. The only postoperative complication was an extensive subcutaneous emphysema, which occurred when the rubber intratracheal tube was removed during the first postoperative day in one animal.

Summary. Anesthesia for lengthy surgical procedures in goats was accomplished by the intermittent administration of sodium pentobarbital into a continuous intravenous (jugular) infusion. Adequate airway was maintained with a low tracheotomy.

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Maintenance of Physiologic Temperatures by Halogenated Corticoid in Adrenalectomized Mice Given Brucella Somatic Antigen.* (21544)

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The temperature reaction of mice to the somatic antigen ("endotoxin") of *Br. melitensis* has been the subject of earlier work from this laboratory(1). During those investigations it was noted, first, that a decrease in rectal temperature below the range of physiologic variation usually preceded by several hours the occurrence of death from endotoxin. Second, in adrenalectomized mice, this hypothermia was induced by lower doses of endotoxin and occurred earlier as compared with intact mice. Third, some protection against the hypothermia was afforded by the simultaneous administration of cortisone acetate or, at higher dose levels, by the administration of desoxycorticosterone acetate. In this report, the protective effects against hypothermia of a corticoid bearing a halogen atom in the 9 α-position will be compared with the corresponding biological activity of cortisone acetate.

Materials and methods. Five groups, composed each of 12 male CBC mice, 2 months of age were used. Purina Fox Chow and tap water were available to the mice *ad libitum* from weaning and throughout the experiment. For 7 days prior to the study the mice were kept singly housed in a room illuminated by artificial light only. A clock-controlled switch turned the lights on at 06:00 and off at 18:00. The mice were adrenalectomized between 08:00 and 12:00 and were tested during the afternoon of the same day. The *Brucella endotoxin* employed was a Boivin-type of antigen(2,1), suspended in saline. Each mouse was injected intraperitoneally with 0.2 mg of endotoxin with or without additional corticoids. The corticoids used were saline suspensions of 9 α-fluorohydrocortisone acetate‡ and of cortisone acetate.‡ Rectal temperatures were measured by means of a thermistor-bridge circuit(3-4) at fifty minute intervals for 6 hours postinjection. Room temperature during testing was 24.5 $\pm$ 1°C.

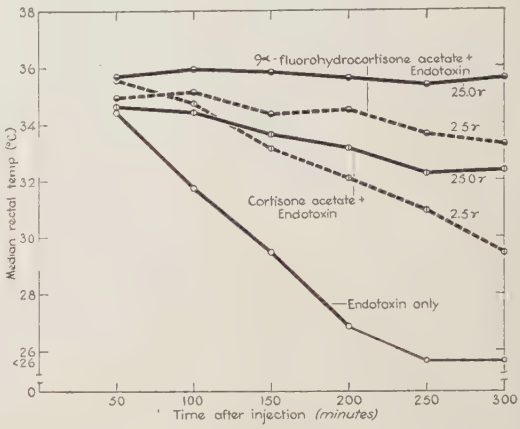

FIG. 1. Rectal temperature of adrenalectomized male CBC mice, given somatic antigen of *Brucella melitensis* (endotoxin) with and without additional corticoid treatment. (12 mice/group.)

Results. The median rectal temperatures of the groups investigated may be seen in Fig. 1. It may be noted from this figure, first, that the rectal temperature of the mice receiving only endotoxin is consistently lower than is the temperature of the treated groups. By 250 minutes after injection, the rectal temperature of the untreated group approaches room temperature; none of these mice had died at this time-point.

By contrast, the temperature of the mice receiving endotoxin with added 25 γ of 9 α-fluorohydrocortisone acetate is maintained within the physiologic range of temperature variation, throughout the entire test-period. At the 2.5 γ dose level, 9 α-fluorohydrocortisone acetate is clearly less potent than at the 25.0 γ dose level, yet its effect compares favor-

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TABLE I. Analysis of Variance Summary.*

Source of variation	DF	SS	MS	F†
(a) Drug effects at 2.5 γ dose level				
Drugs	1	75.4148	75.41	22.1§
Mice on same drug	22	74.9854	3.41	
Times	5	329.3859	65.87	15.8§
Drug × time	5	41.9657	8.39	2.0
Mouse × time	110	457.5428	4.16	
(b) Drug effects at 25.0 γ dose level				
Drugs	1	297.7638	297.76	99.9§
Mice on same drug	22	65.6207	2.98	
Times	5	54.9629	10.99	2.8‡
Time × drug	5	26.5970	5.32	1.4
Mouse × time	110	431.1732	3.92	
(c) Entire experiment (a + b)				
Drug	1	336.4418	336.44	105.1§
Dose	1	127.6536	127.65	39.9§
Dose × drug	1	36.7367	36.74	11.5§
Mice on same drug and dose	44	140.6061	3.20	
Time	5	322.9071	64.58	16.0§
Drug × time	5	67.0548	13.41	3.3§
Dose × time	5	61.4418	12.29	3.0‡
Drug × dose × time	5	1.5079	.30	
Mouse × time	220	888.7160	4.04	

* The breakdown into parts a, b and c exhibits with the analysis of the experiment as a whole also the drug effects at each dose level. The latter were the main points of this investigation.

† Level of significance.

‡ Below 5%.

§ Below 1%.

ably with the effect of 25.0 γ of cortisone acetate.

The results of an analysis of variance, carried out on the data shown in Fig. 1 are summarized in Table I. It is readily apparent that at the two dose levels chosen for comparison, the differences between the effects of cortisone acetate and those of 9 α-fluorohydrocortisone acetate were significant below the one per thousand level. The analysis of the results of the entire experiment (pooled data in Table I) reveals further that for each of the two drugs the increment in dose tested in this study resulted in a significant increment in response ($P < 0.001$).

Discussion. The enhancement of several of the biologic activities of corticoids by the substitution of halogen atoms in the 9 α-position has been the subject of recent reports(5-10). The results reported herein seem to support the view that the substitution of a fluorine atom in the 9 α-position results also in the enhancement of the well known antitoxic activity of corticoids.

Summary. Brucella somatic antigen was given intraperitoneally to five groups of adrenalectomized male CBC mice, kept under standard conditions. Four of these groups received additional treatment consisting of either 2.5 γ or of 25.0 γ of 9 α-fluorohydrocortisone acetate or of cortisone acetate. The rectal temperatures of these mice were measured by means of a thermistor-bridge circuit at 50 minute intervals for a six-hour period. Mice given only the endotoxin showed a progressive decrease of the rectal temperature to the level of the room temperature. Both 9 α-fluorohydrocortisone acetate and cortisone acetate protected against this hypothermia induced by endotoxin, but the former compound was decisively more potent than was the latter. The substitution of a fluorine atom in the 9 α-position of a corticoid seems to be associated with a clear and statistically significant enhancement of antitoxic activity.

1. Halberg, F., and Spink, W. W., to be published.

2. Kabat, E. A., and Mayer, M. M., Boivin-type Antigens in *Experimental Immunochemistry*. Springfield, Charles C. Thomas, 1948, 514.

3. Becker, J. A., Green, C. B., and Pearson, G. L., *Trans. Am. Inst. E. E.*, 1946, v65, 711.

4. Halberg, F., Zander, H. A., Houglum, M. W., and Mühlemann, H. R., *Am. J. Physiol.*, 1954, v177, 361.

5. Fried, J., and Sabo, E. F., *J. Am. Chem. Soc.*, 1953, v75, 2273.

6. Fried, J., *ibid.*, 1954, v76, 1455.

7. Borman, A., and Singer, F. M., *Fed. Proc.*, 1954, v13, 185.

8. Goldfien, A., Beigelman, P. M., Laidlaw, J. C., and Thorn, G. W., *J. Clin. Endocrinol. and Metab.*, 1954, v14, 782.

9. Liddle, G. W., Pechet, M. M., and Bartter, F. C., *Science*, 1954, v120, 496.

10. Callow, R. K., Lloyd, Y., and Long, D. A., *Lancet*, 1954, v267, 20.

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Effects of Transfusions of Lipemic Plasma.* (21545)

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In the present study a natural fat emulsion in the form of lipemic plasma was administered intravenously into a selected group of volunteer patients and the resultant effects determined. The nutritive value of lipemic whole blood and of its derivative lipemic plasma has not been previously explored. Heretofore lipemic blood has been considered clinically unsuitable for transfusion purposes and has been usually withheld at the blood bank because of the following possible contraindications; presence of fatty hemolysins(1), alterations in red cell fragility(2) and formation of free fatty acids in toxic quantities(3). However these latter contraindications have been based on tests made *in vitro* and have not been generally confirmed. Furthermore, fatty chyle, also a natural fat emulsion closely related to lipemic plasma has been infused intravenously into patients without toxic manifestations or other detrimental effects(4).

Method of study. Lipemic plasma was especially processed for this study by the Inter-County Blood Bank, Queens, N. Y. Plasma free of hemoglobin and containing a minimum of 5 g % of total lipids was pooled and bottled 500 ml per flask. Each experiment was started at 8 A. M. after the subject completed a fasting period of 14 hours. Lipemic plasma in units of 500 ml or multiples thereof was infused at 3-5 ml/minute. The subjects' temperature, pulse, respirations and blood pressure were recorded at hourly intervals. Samples of oxalated blood were drawn at hourly intervals for the following determinations; red and white blood cell counts, hemoglobin concentration (Sahli), hematocrit cell volume and sedimentation time (Wintrobe), plasma hemoglobin(5), total lipids(6), chylomicron counts(7), and red cell fragility (Sanford)(8). Also at specific intervals catheterized samples of urine were drawn for routine analysis, for qualitative detection of lipids (Sudan III, alcohol mixture), and for determination of urine hemoglobin (Ortho-tolidin Test). Standard fragility test was performed on red cells derived from pre- and post-infusion samples of blood. Fragility test #1 was made on red cells obtained from recipient prior to infusion of lipemic plasma. Fragility test #2 was performed to determine fragility of erythrocytes exposed to lipemic plasma *in vitro*. The latter test was made on 2 ml of a sample suspension of red cells obtained from pre-infusion blood sample mixed with an equal volume of donor lipemic plasma and incubated for a period of 90 minutes at 37.5°C. The standard test for fragility of red cells was performed. A drop of blood from the red cell suspension obtained from the latter incubation mixture was added to each of a series of test tubes containing 25 drops of saline in concentrations from 0.3% to 0.65% with increments of 0.03%. The dilution at which hemolysis began and ended was noted. Fragility test #3 was made on red cells obtained from subject after transfusion of lipemic plasma. A minimum of three post-infusion tests was done to determine effect of lipemic plasma on circulating red blood cells.

Results. A total of 50 transfusions of lipemic plasma was administered intravenously into 18 subjects. Of these, 8 received a single unit of plasma (500 ml), 6 were infused with 3 units of plasma (1500 ml) and 4 received 2 units of plasma (1000 ml) daily for 3 days. A temperature elevation averaging 3.1°F above normal was noted in association with 4 transfusions. When the febrile reaction appeared in these 4 subjects, it began as the infusion was nearing completion, persisted up to 3 hours and then returned to normal. A febrile reaction did not occur with the other 46 transfusions. No other type of constitutional reaction was observed except for a shaking chill in conjunction with one of the

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TABLE I. Protocol Showing Response of Subject J.L. to Infusion of 500 ml of Pooled Plasma Containing 5.25 g/% of Total Lipids.

	Pre-infusion	Mid-infusion	End of infusion	Post-infusion 4 hr	End of study period
°F	98.6	99.0	98.6	98.2	98.8
White cell count	6,950	12,800	13,450	11,000	8,200
Red " "	4,250,000	3,920,000	3,850,000	4,010,000	4,380,000
Hemoglobin, g/%	12.5	10.5	10.0	12	12.8
Hematocrit cell vol	47	45	43	46	50
Sedimentation time, min.	2	8	8	10	6
Total lipids, g/%	1.56	1.80	1.88	1.82	1.44
Total cholesterol, mg/%	380	330	325	376	402
Plasma Hgb, mg/%	2	2	2	0	0
Chylomicrons count	20	160	172	54	28
	%				
Fragility test 1	B—.45 C—.33	—	—	—	—
2	B—.51 C—.42	—	—	—	—
		%	%	%	%
3	—	B—.51 C—.45	B—.48 C—.39	B—.48 C—.36	B—.48 C—.33

B = Beginning of hemolysis; C = Complete hemolysis.

4 transfusions mentioned above.

Concentration of hemoglobin, number of red cells and hematocrit cell volume showed only the temporary effect due to hemodilution. Sedimentation rate was not altered significantly. In 26 transfusions, a moderate leukocytosis predominantly neutrophilic in character was noted. Total blood lipids rose rapidly during transfusion of lipemic plasma but returned to normal limits within 3 to 4 hours after completion of injection. In 3 cases blood lipids persisted at an elevated level for approximately 6 hours post-infusion. Several partition studies of total lipids failed to show any characteristic trend except for a slight rise in normal fat. Chylomicron counts of peripheral blood usually were found to parallel the values and trends indicated by the chemical tests. Urinalyses made during study period were within normal limits. A lipuria of significance did not occur in any subject. All tests for urine hemoglobin were negative. Data from a typical experiment have been recorded in Table I.

Results of Fragility test #1 were within accepted normal limits in all subjects. Hemolysis in preinfusion blood samples began at 0.45% and was complete at 0.33%. After exposure to lipemic plasma *in vitro* (Fragility Test #2), the erythrocytes of 6 subjects showed an increased susceptibility to hemolysis. Hemolysis in this group began earlier, usually at 0.51%, and was complete at 0.45% or 0.42%. In the immediate post-infusion blood sample and in the 4 hour post-infusion blood sample, erythrocytes exposed to lipemic plasma *in vivo* showed alterations in fragility similar to the *in vitro* tests. At end of study period, fragility of the erythrocytes returned to normal limits in all cases. Free hemoglobin appeared in the circulation plasma and rose to an average of 36.5 mg % within 4 hours post-infusion in the 6 cases. Correlative data on temperature reaction, hemoglobinuria and hemolysis pertaining to 4 subjects have been included in Table II. In the other 2 subjects although hemoglobinemia and increased fragility of red cells were noted, a temperature elevation did not occur. In all the 6 cases manifesting hemoglobinemia, post-infusion urine samples were negative for hemoglobin.

Discussion. Except for temperature reactions which occurred in 8% of all transfusions, lipemic plasma administered intravenously was well tolerated. By contrast, the incidence of reactions to intravenous infusions of artificial fat emulsions ranged about 20%. Post-transfusion temperature elevations seemed to be correlative with hemoglobinemia due to intravascular hemolysis. Thermogenicity of osmotically and chemically

TABLE II. Observations on 4 Patients Having Reactions following the Infusion of Lipemic Plasma.

	Case 1		Case 2		Case 3		Case 4	
	P*	MP†	P	MP	P	MP	P	MP
°F	98.6	101.2	98.8	101.6	98.8	100.8	98.8	100.2
Plasma Hgb, mg/%	2	48	0	54	1	20	0	24
Urine Hgb, mg/%	0	0	0	0	0	0	0	0
	%	%	%	%	%	%	%	%
Fragility test 1	B—.45 C—.39	—	B—.42 C—.45	—	B—.48 C—.42	—	B—.45 C—.42	—
2	B—.51 C—.39	—	B—.57 C—.45	—	B—.54 C—.42	—	B—.51 C—.42	—
3	—	B—.60 C—.51	—	B—.57 C—.48	—	B—.48 C—.42	—	B—.54 C—.45

B = Beginning of hemolysis; C = Complete hemolysis.
* P = Preinfusion. † MP = Maximum post-infusion.

produced hemolysis has been a controversial subject and the relationship of extracorpuscular circulation hemoglobin in the production of reactions has not been determined. Lysis of erythrocytes caused by exposure to lipemic plasma *in vitro* may be assumed to be due to development of a complex hemolytic system composed of one or more undetermined components responsible for the resultant stromatolysis. Hemoglobinuria as a consequence of hemoglobinemia, did not occur because renal threshold of 130 mg % Hgb. was not exceeded. Lipids derived from lipemic plasma and infused into normal subjects were cleared rapidly from blood without excretory loss. Hyperlipemia with delayed clearance in 3 subjects could be accounted for on the basis of the presence of atherosclerosis.

Summary. Eighteen subjects received a total of 50 transfusions of lipemic plasma which was well tolerated. The most significant laboratory tests were associated with fragility of erythrocytes exposed to fatty plasma both *in vitro* and after injection into the circulatory blood. The mechanism for alterations in fragility of erythrocytes remains undetermined but seems to account for the varying thermogenic reaction complicating the use of both natural and artificial intravenous fat emulsions.

1. Johnson, V., Freeman, L. W., and Longini, J., *J.A.M.A.*, 1944, v124, 1250.

2. Creditor, M. C., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 83.

3. Shafiroff, B. G. P., Mulholland, J. H., Roth, E., and Baron, H. C., 1949, v70, 343.

4. Little, J. M., Harrison, C., and Blalock, A., *Surg.*, 1942, vII, 392.

5. Ham, T. H., *Arch. Int. Med.*, 1939, v64, 1271.

6. Bloor, W. R., *J. Biol. Chem.*, 1928, v77, 53.

7. Frazer, A. C., and Stewart, H. C., *J. Physiol*, 1939, v95, 21.

8. Gradwohl, *Clinical Laboratory Methods*, vI, p522, C. V. Mosby.

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Metabolism of β-Methyl-C_5 Fatty Acids by Mitochondria of Rat Liver: Effect of Biotin Nutriture.* (21546)

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Data now available suggest that isovaleric acid undergoes activation, dehydrogenation, and hydration as do straight chain fatty acids, and that the resulting β-hydroxyisovaleryl CoA‡ then fixes CO_2 to form β-hydroxy-β-methylglutaryl CoA, which cleaves to acetoacetate and acetyl CoA(1-5). Metabolism of isovalerate has been shown to occur in slices(1,2), homogenates(3), and soluble extracts(4) from liver. A very slow oxidation of isovalerate by fortified, washed particulate preparations of guinea pig liver has been recorded(6), but the conditions for net oxidation of isovalerate by mitochondria free from other cellular components have not been described. The incorporation of C^{14} from bicarbonate into acetoacetate in the presence of isovalerate has already been shown to proceed much more slowly in biotin-deficient liver homogenates than in those from normal rats(7), but under these conditions no net formation of acetoacetate could be shown even in normal animals. The present work describes conditions favorable to net oxidation of isovalerate and net formation of acetoacetate from isovalerate, 3-methyl-2-butenoate, and 3-methyl-3-butenoate by liver mitochondria from normal rats, and also shows that mitochondria from biotin-deficient rats completely failed to produce acetoacetate from isovalerate and its unsaturated analogs.

Experimental. Reagents were obtained as follows: disodium ATP, Pabst Laboratories; cytochrome c and 65% pure DPN, Sigma; 3-methyl-2-butenoic acid, Farchan; 3-methyl-3-butenoic acid (b.p. 83-85°C at 13 mm Hg), synthesized according to Wagner(8); all other fatty acids, Eastman. The solutions of saturated fatty acids were neutralized with KOH to pH 7. The unsaturated fatty acids were stored at –10°C; solutions were prepared just before use and KOH added only to pH 6. *Mature rats* of both sexes maintained on stock ration of natural feedstuffs were employed in experiments with normal animals. For studies of effects of biotin deficiency, weanling male Sprague-Dawley rats were raised on a biotin-deficient diet(9).§ All rats were allowed access to feed until time of sacrifice. *Analytical methods* employed were acetoacetate(10), inorganic phosphate(11), and nitrogen by micro, direct nesslerization. Mitochondrial suspensions(12) averaging 3 mg N/ml were prepared so that 1 ml was derived from 1 g fresh liver. Each Warburg flask contained 0.5 ml mitochondrial suspension, 6 μM ATP, 22.5 μM $MgSO_4$, 120 μM potassium phosphate buffer, 1.5 μM DPN, 0.03 μM cytochrome c, 6 μM fatty acid substrate (except that 10 μM of *dl*-α-methylbutyrate, and 3 μM of caprylate were used), and 0.15 M KCl to a total volume of 3 ml. Additional components, when present, were added at the expense of KCl at following levels: 10 μM of citrate or glutamate or 5 μM of α-ketoglutarate, 30 μM of sodium malonate, 15 μM of $KHCO_3$. NaOH was used in center well except when bicarbonate was added to the reaction mixture. The final pH at end of 40 minutes incubation was 7.4-7.7, which was optimal for the reaction with isovalerate as substrate. After incubation, usually for 40 minutes, at 30°C, acetoacetate was determined in perchloric acid filtrates of flask contents. Acetoacetate formed was 40 minute value minus a zero time value. When acetone dried powders of mitochondria were used, they were prepared according to Drysdale and Lardy(4). In experiments with Na $HC^{14}O_3$, methods used for degradation of

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‡ Abbreviations used in this paper: coenzyme A (CoA), adenosine triphosphate (ATP), diphosphopyridine nucleotide (DPN).

§ Amounts of vitamins quoted(9) were actually for 10 g of rather than for 100 g of ration.

TABLE I. Requirements for Metabolism of β-Methyl-C_5 Fatty Acids by Mitochondria.

Fatty acid	△ μM acetoacetate*				
	No additions	$KHCO_3$	$KHCO_3$ + citrate	$KHCO_3$ + malonate	$KHCO_3$ + malonate + citrate
None†	.0	.1	.0	.1	.3
β-Methyl acids					
Isovalerate†	.1	1.1	1.8	.5	2.2
3-Methyl-2-butenoate	.4	2.3		.7	3.2
3-Methyl-3-butenoate	.5	2.1		.8	2.6
Other acids‡					
n-Valerate	.9	1.3		1.8	2.3
α-Methylbutyrate	.3	.2		1.0	.9
n-Caprylate§	1.1	1.1		1.7	

* Figures represent values of typical experiments except where otherwise indicated.
† DPN and cytochrome c omitted; these figures are averages of all experiments done under these conditions.
‡ DPN and cytochrome c omitted.
§ Experimental period 25 min.

acetoacetate and counting of radioactivity were those referred to by Plaut(7).

Results. Acetoacetate formation from C_5 fatty acids by mitochondria. Rat liver mitochondria failed to produce appreciable amounts of acetoacetate from isovaleric acid in the standard reaction mixture, whereas *n*-caprylic acid was metabolized readily in this system. It is shown in Table I that metabolism of isovalerate was made possible by addition of bicarbonate. This stimulatory effect of bicarbonate was not unexpected since CO_2 has been shown in isotopic experiments to be incorporated stoichiometrically into the acetoacetate arising from the isopropyl portion of isovalerate(2,3). The requirement for bicarbonate demonstrates the compulsory nature of CO_2 fixation in mitochondrial metabolism of isovalerate. Malonate, added to prevent acetyl CoA oxidation and thus to increase acetoacetate formation, always depressed, but did not prevent completely, the acetoacetate production obtained in presence of bicarbonate. This depression could be overcome by addition of citrate, glutamate, or α-ketoglutarate along with bicarbonate and malonate. Although choline was oxidized nearly half as rapidly as α-ketoglutarate, no stimulation of acetoacetate production from isovalerate was obtained with choline. Since phosphorylation accompanying oxidation of choline has been demonstrated with mitochondria(13), it appears that stimulation by di- and tricarboxylic acids probably cannot be explained solely by the generation of ATP in oxidative phosphorylation. Combination of bicarbonate, malonate and di- or tricarboxylic acid consistently resulted in the greatest yields of acetoacetate from isovalerate. The presence of citrate along with bicarbonate in the absence of malonate always resulted in accumulation of considerable amounts of acetoacetate, since two-thirds or more of the acetoacetate produced during isovalerate metabolism is formed from the isopropyl group by fixation of CO_2(2), and acetoacetate is not further metabolized appreciably by rat liver(14) even when citric acid cycle is operative.

Metabolism of the 2 unsaturated β-methyl-C_5 fatty acids 3-methyl-2-butenoic and 3-methyl-3-butenoic acids, like that of their saturated counterpart, isovaleric acid, proceeded very slowly in the standard system (Table I). Acetoacetate formation from these unsaturated acids also was stimulated by bicarbonate, depressed by malonate in the presence of bicarbonate, and stimulated by citrate in the presence of bicarbonate especially when malonate was present. Experiments have shown that malonate in the reaction mixture with isovalerate prevents resynthesis of ATP; thus at the end of 40 minutes of incubation the increase in inorganic phosphate was over 6 μM in presence of malonate, as compared to 0.5 μM in its absence. The depressing effect of malonate upon acetoacetate formation is not

TABLE II. Fixation of C^{14} from $NaHC^{14}O_3$ Accompanying Metabolism of β-Methyl-C_5 Fatty Acids by Mitochondria.*

Fatty acid	△ μM aceto-acetate	Specific activity, cts/sec/mM	% of added C^{14} fixed
Isovalerate	3.24	5,500	6.6
3-Methyl-2-buten-oate	5.76	5,400	11.5

* DPN and cytochrome c omitted; 3 μM of α-ketoglutarate and 4 μM of $NaHC^{14}O_3$ added/flask. Incubated at 30°C for 60 min.

observed with *n*-caprylic, *n*-valeric, or α-methylbutyric acids (Table I). In extracts of acetone-dried powder of mitochondria dialyzed 3½ hours anaerobically against 0.02 M pH 7.35 tris buffer in the cold, addition of α-ketoglutarate or of malonate had little effect upon isovalerate metabolism. When the extract was dialyzed for 15 hours, no formation of acetoacetate from isovalerate was found, although ability to metabolize *n*-caprylate was unimpaired.

Fixation of C^{14} by Mitochondria. Table II shows that rat liver mitochondria catalyzed fixation of C^{14} from bicarbonate into acetoacetate produced from isovalerate and 3-methyl-2-butenoate, as already reported with liver slices(2), homogenates(3), and soluble systems(4). No appreciable radioactivity was fixed by N-acetyl-S-acetonyl-β-mercaptoethylamine (prepared by reaction of bromoacetone with N-acetyl-β-mercaptoethylamine; composition of 2, 4-dinitrophenylhydrazone, experimental C 44.4, H 4.7, N 19.9, S 8.4 %, calculated C 44.0, H 4.8, N 19.7, S 9.0 %) or by S-acetonyl CoA.

Effects of biotin deficiency. Mitochondria prepared from livers of biotin-deficient rats were not able to produce acetoacetate from isovalerate and its unsaturated analogs in a fortified reaction mixture containing bicarbonate, citrate, and malonate in addition to standard components (Table III). When biotin-deficient animals were injected with 100 μg biotin daily for 3 days, acetoacetate formation was restored. This finding supports the previous report that incorporation of bicarbonate-C^{14} into acetoacetate in presence of isovalerate is greatly depressed in liver homogenates from biotin-deficient rats(7). In contrast to the three β-methyl-C_5 fatty acids, the other C_5 acids investigated, *n*-valerate and α-methylbutyrate, gave rise to acetoacetate in both biotin-deficient and biotin-supplemented groups.

Discussion. Stimulation by bicarbonate of metabolism of the three β-methyl-C_5 fatty acids isovalerate, 3-methyl-2-butenoate, and 3-methyl-3-butenoate, and the requirement for adequate biotin nutriture suggest a common or similar metabolic pathway in their utilization. These data are consistent with the mechanism of isovalerate oxidation reviewed under Introduction. The product of dehydrogenation of isovaleryl CoA would be the acyl CoA derivative of 3-methyl-2-butenoate if α, β-dehydrogenation occurred; however, β, γ-dehydrogenation is also a possibility, in which case the product would be the CoA derivative of 3-methyl-3-butenoate.

Sensitivity of mitochondrial metabolism of β-methyl-C_5 fatty acids to malonate could be explained by ability of malonate to block the citric acid cycle, thus preventing resynthesis of ATP. Oxidation of isovalerate to acetoacetate would generate, per mole of isovalerate, only about 2 moles of ATP, one of which

TABLE III. Effect of Biotin Deficiency upon Metabolism of β-Methyl-C_5 Fatty Acids by Mitochondria.

Fatty acid	Biotin deficient: No. of rats	Biotin deficient: △ μM aceto-acetate range	Biotin inj.*: No. of rats	Biotin inj.*: △ μM aceto-acetate range
None	6	.1- .7	4	.4- .6
β-Methyl acids				
Isovalerate	4	−.3- .3	4	1.8-5.6
3-Methyl-2-butenoate	3	.1- .2	3	1.5-1.6
3-Methyl-3-butenoate	4	−.2- .4	2	2.1-3.7
Other acids				
n-Valerate	4	1.3-2.5	2	2.9-3.4
α-Methyl-butyrate	3	1.5-2.1	1	2.2
n-Caprylate†	1	3.2		

Malonate, citrate, and $KHCO_3$ present in addition to standard components.

* Prior to sacrifice, 100 μg biotin were injected daily for 3 days into biotin-deficient rats; some deficient animals were sham injected with distilled water.

† Experimental period 25 min.; DPN and cytochrome c omitted.

would be required for activation and another for carboxylation reaction(5). In contrast, *n*-caprylate, whose metabolism is uninhibited by malonate, would give a net gain of about 14 moles of ATP per mole of fatty acid. Possibly citrate, glutamate, and α-ketoglutarate stimulate the malonate-blocked system by their conversion to succinyl CoA, which could then transacylate to activate isovalerate.

Summary. 1. In a reaction mixture containing KCl, Mg^{++}, phosphate buffer and ATP, net acetoacetate formation from *n*-valerate, and *n*-caprylate by rat liver mitochondria proceeded readily. Under conditions of our experiments, metabolism of isovalerate, 3-methyl-2-butenoate, and 3-methyl-3 butenoate proceeded only in the additional presence of bicarbonate. Malonate markedly depressed metabolism of the latter three acids which occurred in the presence of bicarbonate. Cooxidation of citrate, glutamate, or α-ketoglutarate overcame this malonate inhibition. Malonate did not decrease the yield of acetoacetate from *n*-valerate, α-methyl-butyrate, or *n*-caprylate. 2. Mitochondria from both biotin-injected rats and biotin-deficient rats produced acetoacetate from *n*-valerate, α-methylbutyrate and *n*-caprylate. The β-methyl fatty acids, isovalerate, 3-methyl-2-butenoate, and 3-methyl-3-butenoate were metabolized by mitochondria of biotin-injected rats but not by mitochondria of deficient rats.

1. Zabin, I., and Bloch, K., *J. Biol. Chem.*, 1950, v185, 117.

2. Coon, M. J., *ibid.*, 1950, v187, 71.

3. Plaut, G. W. E., and Lardy, H. A., *ibid.*, 1951, v192, 435.

4. Drysdale, G. R., and Lardy, H. A., *ibid.*, 1953, v202, 119.

5. Bachhawat, B. K., Robinson, W. G., and Coon, M. J., *J. Am. Chem. Soc.*, 1954, v76, 3098.

6. Muñoz, J. M., and Leloir, L. F., *J. Biol. Chem.*, 1943, v147, 355.

7. Plaut, G. W. E., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 769.

8. Wagner, R. B., *J. Am. Chem. Soc.*, 1949, v71, 3214.

9. MacLeod, P. R., Grisolia, S., Cohen, P. P., and Lardy, H. A., *J. Biol. Chem.*, 1949, v180, 1003.

10. Barkulis, S. S., and Lehninger, A. L., *ibid.*, 1951, v190, 339.

11. Lowry, O. H., and Lopez, J. A., *ibid.*, 1946, v162, 421.

12. Lardy, H. A., and Wellman, H., *ibid.*, 1952, v195, 215.

13. Rothschild, H. A., Cori, O., and Barron, E. S. G., *ibid.*, 1954, v208, 41.

14. Lehninger, A. L., *ibid.*, 1946, v164, 291.

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Regression of Transplanted Rat Lymphoma No. 8 Following Oral Administration of Either 4-(p-Dimethylaminostyryl) quinoline Methiodide or Methochloride.* (21547)

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Haddow, Harris, Kon and Roe(1) have reported on the tumor-inhibitory action of a number of styryl quinoline compounds against Walker rat carcinoma #256. Among them they found that 2-(p-dimethylaminostyryl)quinoline methiodide (Styryl 15) possessed anti-tumor properties; 4-(p-dimethylaminostyryl)quinoline methiodide and 4-(p-dimethylaminostyryl)quinoline methochloride were not studied. The testing of chemical compounds for potential tumor-inhibitory agents is fraught with uncertainty for, even after compounds are selected, there remains the problem of what animal tumor

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TABLE I. Effect of Oral Administration of (A) 4-(p-dimethylaminostyryl)quinoline Methiodide and (B) 4-(p-dimethylaminostyryl)quinoline Methochloride (Each as a 0.3% Concentration in Powdered Purina Fox Chow Diet) on Lymphoma #8 Growing as a Subcutaneous Transplant. 30 rats in each experimental series.

Days of tumor growth before admin. styryl-quinoline	Avg tumor size before treatment, mm	No. of days of treatment	No. of days required for tumor regression	Avg wt of rats, g		
				Before treatment	After tumor regression	30 days following tumor regression
(A) 4-(p-dimethylaminostyryl)quinoline methiodide						
8	26 × 21 × 22	18	10	229	188	235
10	32 × 22 × 20	13	10	199	159	215
12	38 × 22 × 20	12	10	253	219	291
14	25 × 40 × 21	12	9	161	135	187
16	29 × 56 × 27	10	10	202	157	229
(B) 4-(p-dimethylaminostyryl)quinoline methochloride						
10	38 × 22 × 20	11	6	184	150	210
14	42 × 26 × 24	12	10	187	152	220
16	30 × 60 × 30	10	10	201	160	236

might offer the most favorable test object. Rat lymphoma #8, a transplanted tumor growing in the inbred strain in which it originated, proved to be sensitive to the compounds selected for trial. Nine styryl quinolines† were found to affect the course of this lymphoma. Seven of them (2-(p-dimethylaminostyryl)quinoline methiodide and ethiodide, 2-(p-diethylaminostyryl)quinoline methiodide and ethiodide, 4-(p-diethylaminostyryl)quinoline methiodide and ethiodide and 4-(p-dimethylaminostyryl)quinoline ethiodide) showed tumor-inhibitory properties in varying degrees.‡ The present study, however, concerns only 4-(p-dimethylaminostyryl)quinoline methiodide and methochloride.

Lymphoma #8 arose spontaneously in the submaxillary region of a female breeder of the Wistar inbred strain of rats in 1953(2). It has never failed to grow when transplanted subcutaneously in rats of this strain. It metastasizes to the mediastinal, axillary, inguinal and lumbar lymph nodes of the host. The tumor has always been fatal, killing the host animal 18 to 24 days after implantation. The neoplastic cells invade the surrounding subcutaneous tissue and muscles progressively.

Procedure. Three hundred sixty inbred rats (45 to 60 days of age) were implanted subcutaneously by trocar with lymphoma #8. One hundred twenty rats were used as experimental control. The implanted tumors in the remaining 240 rats were permitted to grow for 8 to 16 days before treatment of the host animals. The response of these rats to oral administration of the styryl quinolines (each as a 0.3% concentration in powdered Purina Fox Chow diet) is summarized in Table I, which records the average size of the tumors before treatment, the average weight of the rats before and after treatment, the length of treatment and the time required for complete regression of tumor.

Results. The lymphomas in all treated rats became flabby, rapidly decreased in size and disappeared within 10 days. They did not manifest visible areas of necrosis. All 120 control rats (implanted with lymphoma #8 at the same time as the treated groups) died in 18 to 21 days. The average weight of the tumor-bearing control rats (weighed at the same time as the experimental groups before treatment began) was 194 g; at death it was 212 g. The average weight of the tumors excised from the control rats after death was 61 g. All rats lost weight during treatment, a considerable portion of which was due to the loss of the weight of the disappearing tumor. In the present status of our studies it is difficult to evaluate what influence the actual body weight loss had on tumor regression. Following treatment all rats continued

† These compounds were synthesized in the laboratory of the Department of Chemistry, Carson-Newman College, Jefferson City, Tenn.

‡ Details of these studies are reported in a separate communication.

to gain weight during the observation period. The 240 treated rats remained under observation for 60 days after initial treatment was stopped. All rats survived the first 30 days of this period; in the second 30 days 23 rats died. There was no recurrence of tumor at the original site in any of the treated rats. In the second 30-day period, however, approximately 50% of the surviving rats developed metastatic growths in the axillary or inguinal regions. These regressed completely after resumption of oral administration of the respective styryl quinoline (again as a 0.3% concentration in chow) for 10 days.

Summary. One hundred fifty rats of the Wistar inbred strain bearing #8 lymphomas which had grown for 8 to 16 days following transplantation and had attained sizes ranging from approximately 26 x 22 x 20 mm to 50 x 40 x 22 mm, were fed pulverized Purina chow containing 0.3% of 4-(p-dimethylaminostyryl) quinoline methiodide; and 90 rats bearing #8 lymphomas which had grown for about the same number of days and had reached about the same size, received chow containing 0.3% of 4-(p-dimethylaminostyryl)quinoline methochloride. Complete regression of the original lymphoma occurred in all the rats during 10 to 18 days treatment with the styryl quinoline compounds. There was no recurrence of tumor at the original site during a 60-day observation period following the initial treatment. In the latter 30 days of observation 23 of the 240 treated rats died. In this period approximately 50% of the surviving rats developed metastatic axillary or inguinal growths. All of these metastases regressed completely after a second oral administration of the respective styryl quinoline (again as a 0.3% concentration in powdered chow) for 10 days. One hundred twenty control rats implanted subcutaneously with lymphoma #8 at the same time as the treated rats died in 18 to 21 days. The average weight of the lymphomas excised from the control rats after death was 61 g.

1. Haddow, A., Harris, R. C., Kon, G. A. R., and Roe, E. M. F., *Phil. Trans. Roy. Soc. London A,* 1948, v241, 147.

2. Lewis, M. R., *Science,* 1953, v118, 355.

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Comparative Utilization of Dietary Sr^{90} and Calcium by Developing Rat Fetus and Growing Rat. (21548)

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The early work of Pecher(1) emphasized that there was a close analogy in metabolic behavior of Sr^{89} and Ca^{45} intravenously injected into mice, although about 1.7 times as much Ca^{45} as Sr^{89} was retained. Since that time, the similarity in behavior of these two elements has been stressed, rather than any dissimilarity. The comparative behavior of calcium and strontium in the body may be influenced by many variables. For example: (a) in single dosage studies ion-exchange mechanism may be primarily responsible for skeletal accumulation of radioisotopes, which would tend to minimize observable differences in metabolic behavior of the 2 elements, (b) intravenous studies would to a large degree eliminate the effects resulting from differences in absorption from the G. I. tract, (c) a high strontium to calcium ratio in the diet may lead to an abnormal functioning, as in strontium rickets, (d) nutritional history of the animal may be a determining factor, particularly in relation to adequacy of diet for normal skeletal development, and (e) rate of skeletal growth in relation to timing and duration of a particular study may have a considerable effect upon experimental results.

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The work reported here was designed to answer a specific question: what is the comparative deposition of Sr^{90} and dietary calcium in the newborn and in the skeleton of the rat exposed to continuous ingestion of a normal diet in which carrier-free Sr^{90} has been incorporated? Corollary information was obtained on the contribution of maternal calcium and maternal Sr^{90} to the developing fetus.

Methods. Sr^{90}-labeled feed was prepared as follows: Commercial rat pellets (Rockland, 1.9% Ca) were ground and 500 g mixed with about 500 ml of water to which had been added approximately 200 μc of carrier-free Sr^{90} as chloride. Substantially lower levels of radioactivity would have sufficed for the primary objectives. However, the higher levels facilitated observations on deposition of Sr^{90} in the fetus after dams had been removed from the labeled diet. Each batch was mixed for about 20 minutes employing an ordinary kitchen mixer. The paste was then rolled and cut into squares which were oven-dried at 105°C. The squares were moistened slightly before feeding to the rats. Analyses showed that Sr^{90} was uniformly distributed in the feed and that a reasonably constant Sr^{90}/Ca ratio could be produced over several months of experiment. Highly inbred Wistar rats were used. It was satisfactory to employ ordinary wire mesh cages with hardware cloth flooring arranged so that excreta fell through onto removable trays. Possible contamination of surrounding area with radioactive feed, excreta or dust particles was minimized by construction of a plywood barricade extending from table-top on which cages were placed, to about 2 ft. above cages. The barricade contained a simple sliding door arrangement to allow access to cages. Careful monitoring of area and air during experiment indicated that there was no significantly detectable spread of Sr^{90}. When it became evident by palpation that a female was about ready to deliver the young, the rat was removed to an individual cage for close observation. Thus it was possible to remove the newborn for analysis before suckling when necessary. *Total calcium* was determined on feed, newborn and bones by the usual methods(2). For the

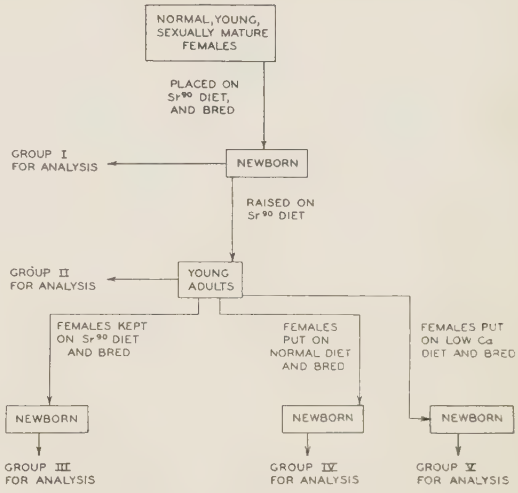

FIG. 1. Experimental plan.

Sr^{90} assay, 10 ml aliquots of ash solution were liquid counted after a 21 day waiting period. The same Geiger counter was used for most part throughout experiment and no significantly large changes of sensitivity were noted during experimental period. Any such changes were unimportant since representative feed and tissue samples were counted in batches on the same day, and thus all samples were subject to the same effect. All values are therefore comparable and expressed in counts/minute of Sr^{90}/mg calcium.

The general plan of the experiment is outlined in Fig. 1.

Comparative utilization for bone growth. Table I presents data on Sr^{90}/Ca ratios of long bones from rats raised entirely on Sr^{90} labeled diet. These rats were the offspring of dams which had been on labeled ration for varying periods of time. As will be indicated later, bones of these rats at birth must have

TABLE I. Comparative Utilization of Sr^{90} and Dietary Calcium by the Rat for Bone Formation. (Feed contained 1481 ± 29 cpm of Sr^{90}/mg Ca.)

Group designation (Fig. 1)	Wt at sacrifice, g	No. of rats	Avg bone value, cpm Sr^{90}/mg Ca
II	70-100	4	438 ± 6.6*
	100-200	4	445 ± 20
	200-300	18	402 ± 13
	300-400	2	453 ± 22
		Weighted avg	417

* Mean ± stand. error.

had a value of about 290 cpm of Sr^{90}/mg of Ca. However, by the time they reached the body weights indicated in Table I, the bone which had been present at birth represented an insignificant contribution to values found at sacrifice. Since feed averaged 1481 cpm Sr^{90}/mg calcium it is evident that overall utilization for bone growth was 1481/417 or about 3.6 in favor of calcium over strontium 90. The ratio was unaffected by size of rat between 70 and 400 g body weight. In a few cases, teeth and bones other than long bones were assayed, always giving the same results. It seemed necessary to make sure that this selective calcium utilization was not a result of experimental procedure. For example, there was the possibility that added Sr^{90} Cl_2 was not assimilated as well as naturally occurring calcium of diet. Therefore, an additional study was carried out, identical in all respects, except that Ca^{45} Cl_2 was used instead of Sr^{90} Cl_2. The feed value averaged 275 ± 10 cpm of Ca^{45}/mg Ca; average of bones from 20 animals which had formed all their skeleton from this feed was 302 ± 7. Thus, the Ca^{45} was utilized to the same extent as dietary calcium under these conditions.

Utilization of maternal Sr^{90} by developing fetus: A direct measurement of the transfer of Sr^{90} from the maternal to the fetal skeleton was obtained by use of females which had been on labeled feed all their lives. These females (Fig. 1), presumably with uniformly Sr^{90} labeled bones, were put on a non-radioactive normal or low calcium diet and then bred (Groups IV and V). Data on Sr^{90}/Ca ratios of newborn are presented in Table II. The ratio averaged 33 cpm of Sr^{90}/mg of Ca for newborn from rats on normal calcium diet (Group IV). There was a considerable spread in values; however, length of time that dams were on unlabeled feed did not appear to produce a trend. These data can be used to estimate how much of fetal calcium is derived from skeleton of the dam and how much from dietary intake. The calculation is based upon a skeletal Sr^{90}/Ca ratio of 417 for mothers, and upon the assumption that calcium and Sr^{90} released from maternal skeleton were differentially utilized for bone development by the factor of 3.6 as already determined for

TABLE II. Sr^{90} Deposition in Newborn from Females that Were Removed from Sr^{90}-Labeled Feed and Placed on Normal or Low Calcium Feed.

(Bones of dams contained 417 cpm Sr^{90}/mg Ca, see Table I.)

Group designation	Day that dams were on unlabeled feed	No. of newborn	Newborn values, cpm Sr^{90}/mg Ca
IV, normal calcium	18	7	17.7
	18	7	55.7
	21	3	38.7
	26	3	62.9
	27	5	26.8
	27	1	71.9
	42	8	32.3
	75	4	11.6
	75	9	15.2
	78	3	13.4
	189	3	16.9
			33.0 ± 6.5*
V, low calcium	26	3	62.9
	49	3	146
			104 ± 42*

* Mean $\pm$ stand. error.

the growing rat. Unpublished results from this laboratory have shown that there is no great difference in absorption of calcium and strontium from the gastrointestinal tract. Thus, the selectivity factor of 3.6 for calcium over strontium must result in large part from a mechanism which is operative after the calcium and strontium enter the circulation. It seems reasonable that this same mechanism would be involved for calcium and Sr^{90} which originate from the maternal skeleton. It can then be calculated by the usual methods of isotope dilution that 28.6% of fetal calcium was derived from the maternal skeleton $\left(\frac{33}{417/3.6} \times 100 = 28.6\% \right)$.

The newborn from dams that had been removed from Sr^{90} labeled diet and placed on very low calcium (0.013% Ca) had a Sr^{90}/Ca ratio of 104 (Group V). Only 2 litters were obtained from this group of animals primarily on account of the stress of the low calcium level. The results are not as reliable, therefore, as for other groups. However, there seems little question that more maternal Sr^{90} was found in the fetal skeleton under these conditions. Considering that practically all of the fetal calcium was derived from the

TABLE III. Comparative Utilization of Sr^{90} and Dietary Calcium by Developing Rat Fetus. (Feed contained 1481 ± 29 cpm Sr^{90}/mg Ca.)

Group designation (Fig. 1)	Date females placed on Sr^{90} feed	Dates newborn collected	No. of litters	No. of newborn	Newborn values, cpm Sr^{90}/mg Ca
I	8/21/53	10/20/53–5/21/54	26	177	278 ± 6.7*
I	1/ 5/54	1/28/54–5/27/54	9	68	314 ± 3.9
III†	Newborn from dams raised entirely on Sr^{90} labeled feed		17	159	300 ± 7.8
				Avg	291

* Mean ± stand. error.

† Dams were offspring of 8/21/53 and 1/5/54 females, which offspring were then raised on labeled feed and bred to give the newborn for which analytical values are listed.

mother, and using average value of 104 cpm of Sr^{90}/mg calcium and the factor of 3.6 it is calculated that skeletons of dams should have had a Sr^{90}/Ca ratio of 374. This is in fair agreement with the experimentally determined value of 417 for similar animals. This provides some measure of support for the assumption that calcium and strontium 90 released from maternal skeleton are utilized by the fetal skeleton in a ratio of 3.6 to 1 respectively.

Comparative utilization by developing fetus: Table III presents data on Sr^{90}/Ca ratios of newborn as compared with this ratio in the feed of the dam. As indicated in Fig. 1, Group I newborn were from normal females which had been placed on the Sr^{90} labeled feed and bred at varying times thereafter. Group III newborn were from dams that had been raised entirely on labeled feed and presumably had a uniform Sr^{90} deposition throughout the skeleton. It is noted from Table III that the overall utilization for fetus development was 1481/291 or 5.1 in favor of calcium over Sr^{90}. It is of interest to examine the value of 291 cpm of Sr^{90}/mg Ca in the fetus to see if it is consistent with values determined for bone growth and calculated for the contribution of the maternal skeleton. Assuming that 71.4% of the fetal calcium is derived from feed with a selective calcium utilization factor of 3.6 it is calculated that each mg of fetal calcium would contain $(0.714)\left(\frac{1481}{3.6}\right)$ or 297 cpm of Sr^{90} contributed by the feed. If the maternal skeleton contained no Sr^{90} at all, then the expected fetus value would be simply 297. If the contributing maternal skeleton contained 417 cpm Sr^{90}/mg Ca, the contribution would be 33 (determined experimentally) which added to the feed contribution of 297 would give a total of 330 cpm Sr^{90}/mg Ca. It can be seen that these calculated values are consistent with those of Table III.

Essentially the same results were obtained under the following 3 time relationships: (a) on animals born as early as 23 days after normal females had been placed on Sr^{90} feed, (b) on animals born as long as 9 months after normal females had been placed on Sr^{90} feed, and (c) on animals born from females which themselves had been on Sr^{90} feed since birth. Any differences among these groups would be difficult to observe experimentally, because the contribution of Sr^{90} by even the fully labeled maternal skeleton is so small, presumably on account of the 3.6 factor. It would appear likely that in 23 days or less, that portion of maternal calcium which is to be transferred to the fetus has already become fully labeled from the dietary intake. This is supported by the work of Pecher and Pecher(3) who showed that the last calcium fixed in the mother's bones was the first to be removed for fetal deposition. Also, the data on the rapidity of incorporation in the skeleton of an isotope such as radiostrontium(4) and the concepts of the ion-exchange mechanism are consistent with the possibilities of early attainment of steady state conditions in the "exchangeable" fraction of the maternal skeleton(5).

Discussion. There seems little question that dietary calcium was preferentially util-

ized over dietary Sr^{90} by the rat under conditions of these experiments. Similar behavior may be implied from values given for natural strontium and calcium contents of sea water and skeletons of marine organisms. For sea water a value of 9.23 atoms of strontium/1000 atoms of calcium has been reported(6); comparative values for skeletons of marine organisms have ranged from 2.4 to 3.5(7). These results give a selectivity of 2.6 to 3.8 for preferential utilization of calcium over strontium. Menzel(8) has reported a preferential utilization by plants of 2.5 for soil calcium over added Sr^{89}.

There is as yet no clear evidence as to the means by which the rat discriminates against strontium in favor of calcium. Other studies in this laboratory(9) primarily with cattle have indicated that: (a) Ca^{45} and Sr^{90} entering blood were diluted, exchanged into extravascular fluid, soft tissue and bone compartments at essentially the same rate and to the same degree; (b) absorption from the gastrointestinal tract after ingestion was not greatly different for Ca^{45} and Sr^{90}; (c) ingested Ca^{45} was preferentially secreted into cow's milk by a factor of 6-10 over ingested Sr^{90}; (d) there were no great differences in deposition of ingested Ca^{45} and Sr^{90} in the hen's egg; and (e) Sr^{90} was preferentially secreted in urine as compared with Ca^{45}.

It appears that the preferential excretion of strontium in the urine may account in considerable measure for the overall preferential utilization of calcium over strontium.

Summary and conclusions. Continuous feeding experiments with rats on a Sr^{90} labeled feed indicated that: (a) dietary calcium was utilized for bone growth by a factor of 3.6 over Sr^{90}; (b) about 28.6% of the fetal calcium was derived from maternal calcium; (c) dietary calcium was utilized for bone formation in the developing fetus by a factor of 5.1 over Sr^{90}; (d) this high factor for the fetus was accounted for on the basis that calcium and Sr^{90} released from the maternal skeleton were deposited in the fetus with the same selectivity of 3.6 for calcium as was observed for the growing rat.

1. Pecher, Charles, PROC. SOC. EXP. BIOL. AND MED., 1941, v46, 86.

2. Comar, C. L., Hansard, Sam L., Hood, S. L., Plumlee, M. P., and Barrentine, B. F., *Nucleonics,* 1951, v8, 19.

3. Pecher, Charles and Pecher, Jacqueline, PROC. SOC. EXP. BIOL. AND MED., 1941, v46, 91.

4. Comar, C. L., Lotz, W. E., and Boyd, G. A., *Amer. Jour. Anat.,* 1952, v90, 113.

5. Neuman, W. F., and Neuman, M. W., *Chem. Rev.,* 1953, v53, 1.

6. Odum, Howard T., *Science,* 1951, v114, 211.

7. ———, *ibid.,* 1951, v114, 407.

8. Menzel, R. G., *Soil Science,* 1954, v77, 419.

9. Comar, C. L., *et al.,* Oak Ridge Operations Report 110, April 1954.

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Cytological Response to Infectious Hepatitis Specimens in Embryonated Eggs.* (21549)

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It has been noted(1) that influenza and mumps virus infections in embryonated eggs were associated with increased numbers of histiocytes in the allantoic fluid (AF). When this reaction was quantitated, the cytological response to these asymptomatic viral infections could be correlated in serum neutralization and in infectivity dilution end points with the results attained through use of the hemagglutination phenomenon.

Although serums from acute cases of infectious hepatitis induced no overt alteration in the embryonated egg it has recently been

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observed that inoculations with such specimens were subsequently associated with increased numbers of histiocytes in the AF.

Methods. Bacteria-free serum specimens were inoculated (0.1 ml) into the allantoic cavity of 7 day embryonated eggs. The shell openings were sealed with collodion and the eggs were subsequently incubated at 35°C for 4 days. The eggs were then stored in the refrigerator overnight. The shell over the air sac was cut off and the clear AF was aspirated with a 10 ml syringe and 19 gauge needle. The AF from 4-5 eggs was pooled and 25 ml aliquots were centrifuged at 4000 rpm for 20 minutes. The supernatant fluid was quickly decanted and the sediment was resuspended in the residual fluid. A loopful (approximately 0.01 ml) of the sediment was smeared on a glass slide over an area of 1 square cm. After drying at room temperature and staining with Wright's stain, the air-dried smears were examined microscopically (5X ocular and 100X objective). The total number of histiocytes in 100 consecutive microscopic fields were recorded from duplicate smears. The procedure outlined above was followed with serum specimens from acute cases of infectious hepatitis† and from 3 of the same cases collected during convalescence. Control inoculations included serums from aseptic obstructive jaundice cases, and acute IH serums inactivated by heat, and normal AF from 11 day embryonated eggs. All serum specimens were stored at –20°C. The effect of *antiserums* on the cytological response in AF was determined by mixing equal amounts of heat-inactivated serum and the histiocyte promoting agent either in acute IH serum or in AF being serially passaged in eggs. The mixtures were incubated at 37°C for 30 minutes and inoculated (0.2 ml) into 7 day embryonated eggs. These eggs were thereafter processed according to the technic described above. Serum neutralization thus studied included commercial gamma globulin, baseline and 2 week serum specimens from chickens inoculated with the various lines of

† Including Akiba strain IH kindly supplied by Dr. W. J. Bashe, Children's Hospital, Philadelphia, Pa.

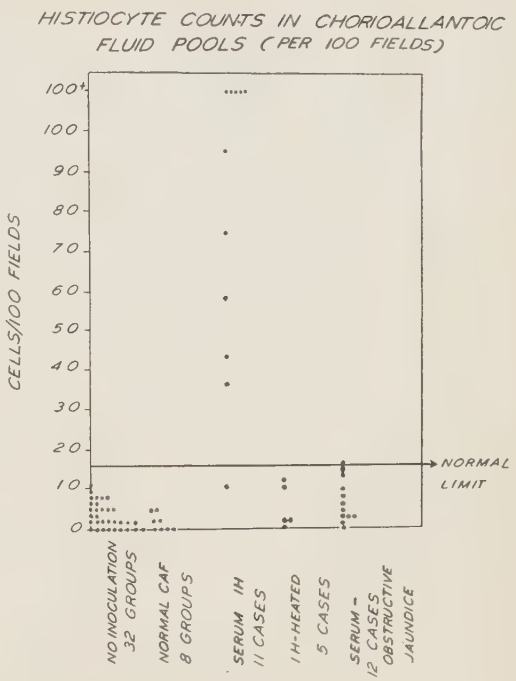

FIG. 1.

IH being studied, and convalescent serum from 3 donors of the original IH serum specimens.

Results. AF from uninoculated eggs and from eggs previously inoculated with normal AF contained histiocytes ranging from 0 to 14 per 100 fields. Icteric serums from 12 cases of obstructive jaundice did not engender histiocytes in excess of this number (Fig. 1). Serum specimens from 11 acute IH cases engendered high histiocyte counts in 10 instances (5 specimens exceeding 100). When these serum inocula were heated at 61°C for 30 minutes the histiocyte counts in AF were

TABLE I. Effects of Gamma Globulin on Histiocyte Response to IH Agents.

IH agent	Histiocyte count*	
	Agent alone	Agent + γ globulin
K Serum	106	12
AP "	77	12
J "	28	9
MC "	106	19
AF Akiba†	120	12
AF AP	55	12

* No. of histiocytes/100 oil immersion fields.
† Egg passage.

TABLE II. Interrelationships among Egg Passaged IH Strains.

Source of IH agent	Antiserum prepared in chickens from: Molis	Snyder	AP	Akiba
Molis	12 (61)*	7 (35)	6 (35)	9 (61)
Snyder	11 (50)	1 (41)	10 (140)	3 (29)
AP	11 (86)		7 (64)	9 (194)
Akiba	8 (31)		1 (23)	3 (50)

* 2 wk antiserum (baseline serum)—No. of histiocytes/100 oil immersion fields.

within the normal limits noted above and normal limits were obtained with IH convalescent serums. The convalescent serum specimens were collected 26 days to 3 months after onset of the acute episode.

Gamma globulin suppressed the histiocyte exudative effect of acute IH serum and of one AF passage stemming from them (Table I). Serum from chickens previously inoculated intravenously with the latter AF fluids, exerted a histiocyte suppressive effect. Serums from such chickens also neutralized the exudative effect of AF fluid of heterologous origin, thus suggesting some interrelationship (Table II). Of three IH cases studied, all developed properties in their convalescent serums which neutralized the exudative effect of the IH agents noted above.

Summary. Serums from 10 acute cases of infectious hepatitis were inoculated into the allantoic cavity of embryonated eggs. The allantoic fluids from these eggs contained numbers of histiocytes far in excess of the limits detected in uninoculated eggs, and in eggs inoculated with heat-inactivated serums and with icteric serums from cases of obstructive jaundice. This histiocyte promoting factor could not be demonstrated in serums from convalescent patients. No bacteria could be associated with this cellular response. Histiocyte counts engendered through inoculations with acute IH serums were returned to normal limits by gamma globulin, by serums experimentally produced in chickens, and by serums from 3 IH-convalescent patients.

1. Pollard, Morris, and Diserens, L. T., *Science*, in press.

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Stimulatory Effect of Calcium on Growth of *Lactobacillus fermenti.** (21550)

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The importance of calcium for the growth of certain microorganisms has been reported by several investigators. Chattaway, Happold and Sanford(1) demonstrated that acid production by *Lactobacillus casei* was increased by calcium in the media. Meinke and Holland(2) presented evidence to show that there is a calcium requirement for *Lactobacillus delbrueckii.* Camien and Dunn(3) reported that calcium was required by *Lactobacillus casei* when grown on media low in serine. When this paper was near completion, Eades and Womack proved that calcium is definitely a growth stimulant for *L. casei,* strain 7469(4).

In seeking a suitable microbiological method for the study of growth factors in fish meal, and fish solubles, it was observed that acid extracts of crab and fish meals would stimulate the growth of *Lactobacillus fermenti* in the presence of an optimal concentration of thiamine. Since the crab and fish meals used contained considerable calcium, it is the subject of this paper to demonstrate that calcium is a stimulatory factor for the growth of *L. fermenti.*

Methods. Lactobacillus fermenti, strain 36 (ATCC 9338) was used as the test organism

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TABLE I. Stimulatory Effect of Various Substances on Growth of *L. Fermenti* 36. Basal medium contg. 20 mg maltose(6) + 0.5 γ thiamine/tube.

Substances added	Amt/10 ml, mg	Turbidity reading, Klett units	Growth increase, Klett units
(A) Crab meal ext. *(Cancer magister)*	0	227	—
	5	246	19
	15	261	34
Turbot meal ext. *(Atheresthes stomias)*	0	223	—
	5	238	15
	15	250	27
Dover meal ext. *(Microstomus pacificus)*	0	223	—
	5	240	17
	15	245	22
Rock fish meal ext. *(Sebastodes* sp.*)*	0	223	—
	5	241	18
	15	246	26
(B) Crab meal ext. (75% ethanol ppt.)	0	227	—
	5	243	16
	15	254	27
(C) Calcium lactate	0.0	249	—
	0.5	265	16
	1.0	270	21
	2.0	279	30
	3.0	284	35
Same medium without maltose(5)			
Calcium acetate (as Ca)	0	186	—
	0.01	194	8
	0.05	201	15
	0.10	209	23
	0.20	217	31
	0.40	218	32

in this study. The Sarett and Cheldelin procedure(5) and the modified method described by Fang and Butts(6) were employed. The extent of growth was determined by measuring the turbidity after 16 hours of incubation at 37°C with a Klett-Summerson photocolorimeter using a 540 mu filter. The results are expressed as Klett units. Meal extracts were prepared by digesting five g of meal with 75 ml 0.1 N H_2SO_4 in a boiling water bath for 30 minutes, with frequent additions of a few drops of 20% H_2SO_4, if necessary, to maintain the acidity. The digest was cooled and made to 100 ml, well mixed, and filtered. Fifty milliliters of the filtrate were neutralized with dilute NaOH to pH 6.5 and diluted to 100 ml. Each ml of the final solution represents an extract from 25 mg of meal. In the following experiments the amount of extract added to the medium was expressed in mgs of original meal.

Results. The growth rate of *L. fermenti* increased as the thiamine level in the medium increased. It was found that with the addition of an acid extract of crab meal, or the fish meals tested, to media containing optimal amounts of thiamine, the test organism would invariably show a heavier growth, (Table I, A). Ethanol (95%) was added to a quantity of neutralized crab meal extract to give a final concentration of 75% ethanol. The resulting precipitate was filtered and washed with 75% ethanol. This alcohol precipitate was found to be responsible for stimulating the growth rate of the *L. fermenti* (Table I, B). The alcohol precipitate was insoluble in alkaline solutions and soluble in acid. The alcohol insoluble material when dissolved in acid could be precipitated by a solution of ammonium oxalate. These properties are very similar to those possessed by some calcium salts. In order to demonstrate the growth stimulating activity of calcium salts, various levels of calcium lactate or calcium acetate were incorporated in the test media. The results indicate that both calcium salts

stimulated the growth of the *L. fermenti,* (Table I, C). Calcium sulfate gave similar results. The calcium added was limited to a maximum of 0.6 mg per tube since greater concentration of calcium resulted in partial precipitation of the medium during steam sterilization. Sodium lactate, when added to the test media, had no effect on growth.

Table II shows that in media containing low levels of thiamine (0-0.02/10 ml) insignificant growth stimulation was obtained regardless of quantities of calcium added. But with higher levels of thiamine, the addition of calcium salts greatly stimulated the growth rate of *L. fermenti.*

Another experiment was conducted to eliminate the factor of the thiamine content of the crab meal. A sample of crab meal was extracted with 0.1 N H_2SO_4. An aliquot of the extract was treated with sodium bisulfite at pH 5.2 to destroy thiamine, and then the excess sodium bisulfite was removed by hydrogen peroxide and brought to pH 6.5 with NaOH. Various quantities of the bisulfite treated crab meal extract were used and its growth stimulating effect compared with that of untreated crab meal extract, (Table III). It is evident that crab meal contained very little thiamine since both the sodium bisulfite treated and untreated extracts behave very much the same. In media containing low levels of thiamine, both extracts showed little effect on the growth of the test organism. However, when the thiamine level increased, both extracts stimulated growth.

TABLE II. Effect of Calcium Added to Media Containing Different Amounts of Thiamine on Growth of *L. fermenti* 36.

Ca acetate (as Ca, mg/tube)	Turbidity reading (Klett units) — Thiamine, γ/tube					
	.0	.01	.02	.03	.05	.10
Basal medium(5)						
.0	48	56	63	67	87	110
.1	48	57	64	69	91	120
.2	50	58	66	71	99	125
.3	52	59	66	72	98	131
.4	53	62	70	75	107	140
.6	58	66	70	75	107	147
Basal medium + 20 mg maltose/tube(6)						
.0	50	63	73	83	125	168
.01	50	63	73	84	130	179
.05	50	63	73	85	131	182
.10	52	63	74	86	135	191
.20	52	66	75	87	138	202
.40	58	73	80	91	139	205
	Thiamine, γ/tube					
	.0	.1	.2	.3	.5	1.0
Basal medium(5)						
.0	46	127	133	143	150	
.1	46	132	154	155	167	
.2	46	136	163	160	169	
.3	46	142	168	172	166	174
.4	51	155	176	180	177	187
.6	57	155	178	185	187	203

TABLE III. Effect of Sodium Bisulfite Treated Crab Meal Extract on Growth of *L. fermenti* 36.

Crab meal extract, mg/tube	Turbidity reading (Klett units) — Thiamine, γ/tube					
	.0	.01	.02	.03	.05	.10
$NaHSO_3$ treated	Basal medium(5)					
0	48	65	77	90	106	140
5	49	66	79	92	119	159
15	51	67	82	99	125	172
Untreated						
0	48	65	77	90	106	140
5	51	68	82	96	119	154
15	50	68	78	99	132	176

Summary. From the data shown in these experiments, it was demonstrated that calcium salts or calcium derived from fish or crab meal stimulated the growth of *L. fermenti* in the presence of optimal amounts of thiamine.

1. Chattaway, F. W., Happold, F. C., Sanford, M., *Biochem. J.,* 1943, v37, 298.

2. Meinke, W. W., and Holland, B. R., *J. Biol. Chem.,* 1950, v184, 251.

3. Camien, M. N., and Dunn, M. S., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 564.

4. Eades, Jr., C. H., and Womack, M., *J. Bacteriol.,* 1953, v65, 322.

5. Sarett, H. P., and Cheldelin, V. H., *J. Biol. Chem.,* 1944, v155, 153.

6. Fang, S. C., and Butts, J. S., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 463.

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Effect of Anticoagulants on Local Shwartzman Reaction. (21551)

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Many agents have been tested for their effect on the local Shwartzman reaction in efforts to elucidate the mechanism of this phenomenon. Almost complete inhibition of the local hemorrhage and necrosis was obtained when large doses of heparin were given during the period of development of the hemorrhagic-necrotic reaction(1,2). This finding is consistent with the histopathological observation that soon after the provocative injection, thrombosis of the smaller blood vessels occurs at the prepared sites(3,4). Heparin, however, has specific pharmacologic actions(5-7) other than inhibition of blood clotting, and additional information may be derived from investigation of the effect of other anticoagulants on the local Shwartzman reaction. To date, bishydroxycoumarin, which failed to prevent hemorrhage and necrosis in 5 of 6 rabbits(8) has been the only other anticoagulant tested.

This report deals with the effect on this reaction of 3 other anticoagulants, and suggests that Tromexan, ethyl biscoumacetate, NNR, which inhibits blood clotting differently from heparin, can block the local Shwartzman reaction.

Materials and methods. Rabbits of mixed breeds, colors and sexes, weighing 1.5 to 2.5 kg were used. Fresh animals were used for each experiment. Prior to giving the preparatory dose, hair was removed from the abdominal skin with electric clippers. The Shwartzman-active material employed was Lot No. P-35 of a polysaccharide prepared by Perrault and Shear(9) from *Serratia marcescens*. The polysaccharide, and all other parenterally administered drugs, was dissolved in sterile pyrogen-free 0.9% saline. The intradermal (preparatory) dose was 125, 250, and 500 μg in 0.25 ml saline in three different abdominal sites in each rabbit. In a fourth site 0.25 ml saline served as a control. The intravenous (provocative) dose, given 24 hr later, was 800 μg in one ml. The reaction was considered positive when purple-black areas of hemorrhage and necrosis developed at one or more of the prepared sites within 6 hr and persisted for 12 hr. In this study 90% of rabbits which received the polysaccharide according to the above schedule, without anticoagulant, yielded a positive reaction. *Heparin*† was given intravenously in a dose of 30 mg/kg, 5 minutes before the provocative dose, then again 2 hr and 4 hr later. Clotting time, on ear vein blood, was done with the capillary tube method just prior to the second dose of heparin. At this dose level of heparin the clotting time was uniformly more than one hr. Chlorazol fast pink‡ (CFP), an anticoagulant azo dye, was given intravenously in a dose of 90 mg/kg, 5 min. before, and again 3 hr. after the provocative dose. The clotting time, just prior to the second injection of this agent, was under 30 min. in half the animals and under 60 min. in the others. All rabbits given this dose of CFP showed considerable prostration. Mepesulfate◊, a synthetic pectic acid derivative(10), was administered in a dose of 75 mg/kg intravenously 5 min. prior to the provocative dose, followed 2 hr later by 50 mg/kg. These animals also showed toxic signs similar to those receiving CFP; apparently both of these anticoagulants had been given at or near the maximum tolerated dose. Clotting time in this series, just prior to the second dose of mepesulfate, ranged from 15 to 30 min. in 60% of the animals, and to less than 60 min. in the others. *Tromexan,*‖ an anticoagulant similar to bishydroxycoumarin but with a

* National Institutes of Health, Public Health Service, U. S. Department of Health, Education and Welfare.

† Hynson, Westcott, and Dunning.

‡ National Aniline, Lot No. 12940.

◊ Kindly supplied by Hoffmann-LaRoche.

‖ Kindly supplied by Geigy Pharmaceuticals.

TABLE I. Effect of Anticoagulants on Incidence of Shwartzman Reaction in Rabbits.

Anticoagulant	Rabbits (No.)	Animals with positive reactions (%)	Chi square*
0	60	90	—
Heparin	12	17	27
Chlorazol fast pink	6	100	0
Mepesulfate	5	100	0
Tromexan			
Pro. time, <25 sec	13	53	8
" " , >25 sec	20	30	26

* Each treated group compared with untreated group by the chi square method. Chi squares larger than 6.6 indicate $p < 0.01$ and are considered significant.

more rapid onset of action(11), was prepared as a 5% solution in 2.6% aqueous Na_2CO_3. It was administered by stomach tube either in a single dose of 0.75 g 24 hr before, or in two equally divided doses (total of 0.9 or 1.0 g) 36 and 24 hr before the provocative dose of the Shwartzman agent. Prothrombin time was used as the index of effectiveness of tromexan since clotting time, as used for the other anticoagulants, does not accurately measure the depression of prothrombin levels achieved by tromexan administration. Prothrombin time was done in duplicate by a one stage method with commercial preparation of calcium and thromboplastin¶ according to the manufacturer's instructions. In control determinations on 43 rabbits the mean prothrombin time, with its standard error, was 7.7 ± 0.5 sec. In the tromexan-treated rabbits, prothrombin times were done prior to administration of the tromexan and 2 hrs after the provocative dose of the polysaccharide. Determinations done at comparable times in a series of 4 control rabbits showed no significant change in prothrombin time during the Shwartzman reaction.

Since the prothrombin times varied considerably in the tromexan-treated rabbits, the data for these animals were classified into two groups according to the prothrombin time at 2 hrs; the dividing line was 25 sec. Mean prothrombin time with the standard error, during the Shwartzman reaction, was 20 ± 1 sec. for the 13 rabbits in the under 25 sec. group, and 43 ± 6 sec. for the 20 rabbits in the over 25 sec. group.

¶ Simplastin, Warner Chilcott.

Results. As seen in the Table, pronounced inhibition of the Shwartzman reaction was obtained with heparin, in agreement with previous reports. In this heparin experiment the clotting time was consistently above one hour. When less heparin was given, and the clotting time was less markedly prolonged, such inhibition of the Shwartzman phenomenon was not obtained(1,2). In the above groups with CFP and mepesulfate no protection against the hemorrhage and necrosis was noted, and most of these animals had a clotting time of 30 minutes or less. No attempt was made to increase the clotting time by increasing the dose of the CFP or mepesulfate because of the toxicity of these compounds. The observed lack of protection with CFP and mepesulfate may have been a consequence of a lack of effective anticoagulation. Significant protection against the dermal hemorrhage and necrosis was afforded by tromexan. Inhibition of this reaction appeared to be related to depression of the prothrombin levels. These data support the thesis that a high enough degree of anticoagulant action inhibits the Shwartzman phenomenon and that thrombosis may be one of the important factors in the development of the hemorrhagic-necrotic reaction.

Summary. 1. Heparin and tromexan, when given in adequate dosage, inhibited the local Shwartzman reaction induced with a polysaccharide from *S. marcescens*. Two other anticoagulants, chlorazol fast pink and mepesulfate, did not produce the requisite prolongation of clotting time, and failed to inhibit the Shwartzman reaction. 2. These observations are consistent with the thesis that thrombosis may be one important factor in the development of the local Shwartzman reaction.

1. Good, R. A., and Thomas, L., *J. Exp. Med.*, 1953, v97, 871.

2. Cluff, L. E., and Berthrong, M., *Bull. Johns Hopkins Hosp.*, 1953, v92, 353.

3. Stetson, C. A., *J. Exp. Med.*, 1951, v93, 489.

4. Thomas, L., *Ann. Rev. Physiol.*, 1954, v16, 467.

5. Wells, J. A., Dragstedt, C. A., Cooper, J. A., and Morris, H. C., PROC. SOC. EXP. BIOL. AND MED., 1945, v58, 57.

6. Anfinsen, C. B., Boyle, E., and Brown, R. K., *Science*, 1952, v115, 583.
7. Jaques, L. B., *Ann. Rev. Physiol.*, 1954, v16, 175.
8. Rothfeld, B., Williams, C. H., Thaler, W. J., and Rodas, J. M., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 5.
9. Perrault, A., and Shear, M. J., *Cancer Research*, 1949, v9, 626.
10. Mangieri, C. N., Engelberg, R., and Randall, L. O., *J. Pharmacol.*, 1951, v101, 25.
11. Burke, G. E., and Wright, I. S., *Circulation*, 1951, v3, 164.

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Effect of Isonicotinic Acid Hydrazide on Niacin and Pyridoxine Metabolism in Rats. (21552)

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The effective treatment of experimental and human tuberculosis with isonicotinic acid hydrazide (INH) has stimulated the search for the mechanism of its biological activity. Structural relationships between INH, niacin and pyridoxine suggested that the antituberculous drug might act as an antimetabolite against either of those vitamins. Thus McConnel and Cheetam(1) reported relief with niacin and B-complex in a case of pellagra which developed during INH treatment. The neuritis occurring in 40% of patients undergoing INH therapy may yield to pyridoxine administration(2). Ungar *et al.*(3) have noted recently testis and thymus involution and growth retardation in rats on high doses of INH. Our studies described here indicate that INH does not interfere with niacin metabolism and that the toxic effects of INH in rats on a B_6-deficient diet can be prevented with pyridoxine.

Materials and methods. Weanling white rats of both sexes were used in these studies. Food and water were supplied *ad libitum*. The basal diet contained 82% sucrose, 10% casein, 4% salts(4), 3% cottonseed oil, 0.5% choline, 0.4% cystine, and supplied the following vitamins in mg quantities per 100 g: thiamin HCl, riboflavin and pyridoxine, 1.0 each; calcium pantothenate, 6.0; inositol, 15.0; biotin and folic acid, 0.02 each; and vit. K, 0.10. Vit. A and D in unitage of 200 and 20 respectively in the form of Meads Standardized Cod Liver Oil were given by dropper twice weekly. For the pyridoxine experiments, the diet was modified as follows: the level of casein was increased to 25% at the expense of the sucrose, choline was reduced to 0.2%, and cystine and pyridoxine were omitted. DPN was analyzed by the fluorometric assay of Robinson *et al.*(5); and xanthurenic acid (XA) was measured in urine colorimetrically(6).

Results. The data in Table I indicate that INH, at a level of 50 mg % in the diet, did not suppress growth, deplete the liver of pyridine nucleotides, or influence the excretion of N^1-methylnicotinamide (NMN) following an injection of l-tryptophan. In addition, INH was without effect on the methylation of nicotinamide, since equal quantities of NMN were excreted in groups B and D during the control period.

Since previous studies by Mann and Quastel(7) had demonstrated that nicotinamide, but not nicotinic acid, prevented the enzymatic degradation of DPN, it was of interest to determine whether INH also was capable of inhibiting this reaction. Freshly prepared rat liver homogenates suspended in 2% nicotinamide, 2% INH, or saline were incubated at room temperature for 30 minutes and then analyzed for pyridine nucleotides. The average values for duplicate samples were 600 μg DPN per gram of liver in the homogenate incubated with nicotinamide, and less than 100 μg DPN per gram in the INH and saline suspensions.

The inhibitory action of INH on pyridoxine was demonstrated in the following experi-

TABLE I. Effect of Isonicotinic Acid Hydrazide on Growth, Liver Pyridine Nucleotides, and Excretion of N'-Methylnicotinamide in Rats on Niacin-Low Ration. Average values for 4 rats in each group.

Group	Supplement to diet	Wt gain, g/wk, for 4 wk	72 hr excretion of NMN, μg Control	72 hr excretion of NMN, μg 25 mg l-tryptophan*	Pyridine nucleotides, μg/g liver†
	mg %				
A	0	7	13	178	456
B	5 niacin	16	240	420	610
C	50 INH	5	19	190	678
D	5 niacin + 50 INH	15	250	435	677

* Tryptophan injected on 32nd day.
† Rats sacrificed at end of 5th wk and samples of liver taken for DPN analysis.

ment, in which 4 groups of 10 rats each were used. The basal diet, lacking in pyridoxine, allowed for an average gain of 10 g per week for 4 weeks. The addition of 2 mg % pyridoxine to the basal diet resulted in excellent growth, averaging 26 g. When the basal diet was supplemented with 100 mg % INH, the rate of growth was depressed to 1.7 g per week; no other physical signs of pyridoxine deficiency were evident. To demonstrate that the growth inhibition of the INH-treated rats was due to a defect in the normal utilization of pyridoxine and not to a toxic dose of the drug *per se,* the animals in the fourth group received both pyridoxine (2 mg %) and INH (100 mg %) in the diet. Pyridoxine completely counteracted the growth retarding action of INH.

To eliminate variations caused by unequal food consumption and absorption of INH from the gut, rats fed the basal pyridoxine-deficient ration were given intraperitoneal injections of the drug daily. Table II shows that the rats in Groups 2 and 3 receiving 2.5 and 5.0 mg of INH respectively lost weight as compared to the control animals in Group 1. When 10 mg of INH were injected into the rats in Group 4, there was an immediate weight loss and the average time of survival was 9 days. Within a few days a syndrome characterized by convulsion and difficult respiration developing within 1 hour after injection of INH was evident in most of the animals. As the data for group 5 indicate, the harmful effects of 10 mg of INH were completely nullified by the simultaneous administration of 200 μg of pyridoxine.

TABLE II. Effect of Pyridoxine on Growth and Survival* of Rats Given Isonicotinic Acid Hydrazide Intraperitoneally. Six rats in each group. Experiment terminated after 4 wk.

Group	Treatment	Wt gain, g/wk
1	.2 ml saline	12
2	2.5 mg INH	6.5
3	5 " "	4.8
4	10 " "	—
5	10 " " + 200 μg B_6	23

* No mortality was observed except in Group 4; in this group the animals lived an avg of 9 days, with mortality spread over a period lasting from third to sixteenth day.

The comparative activities of desoxypyridoxine and INH as inhibitors of pyridoxine were examined. Rats weighing 100-150 g were used in this experiment, otherwise the diet and technics were similar to those used previously. The excretion of XA, an abnormal metabolite of tryptophan which is found in the urine of B_6-deficient rats, served as the index of pyridoxine deficiency. After one week on the basal pyridoxine deficient ration plus the supplements indicated in Table III, 25 mg of l-tryptophan were given intraperitoneally and the 48 hour urine specimens collected and analyzed for XA. The results obtained could not be predicted from our previous experiments. The rats in group K which were treated with 10 mg INH daily excreted significantly less XA than the control Group J and much less than the animals in Group M who were given desoxypyridoxine. Therefore, while the excretion of XA is extremely sensitive to the lack of dietary pyridoxine or the desoxypyridoxine, a highly active antimetabolite of B_6, it is not markedly affected by INH treatment.

TABLE III. Excretion of Xanthurenic Acid in Rats Treated with Isonicotinic Acid Hydrazide and Desoxypyridoxine. Average values for 4 rats in each group.

Group	Diet	48 hr excretion of xanthurenic acid, μg	
		Control	20 mg l-tryptophan
J	Basal (pyridoxine deficient)	269	474
K	Basal + 10 mg INH I.P.	86	159
L	Basal + 10 mg INH & 200 μg pyridoxine I.P.	<10	<10
M	Basal + 50 mg % desoxypyridoxine	—	1750

Discussion. A study was made of the inhibitory effect of INH on several well established pathways of niacin metabolism. In none of these did the large amount of INH given function as an anti-metabolite of niacin. Zatman *et al.*(8,9) recently demonstrated the presence of a DPN-ase in human and animal tissues which catalyzes the exchange reaction between added nicotinamide or INH and the nicotinamide moiety of DPN; this explains the well-known inhibitory effect of nicotinamide on this enzyme. In the *in vitro* DPN-ase experiment, it is conceivable that the inactivity of INH might be attributed to the formation of the INH analog of DPN which would not be measured in the fluorometric assay. In the intact rat, however, the failure of INH to influence the concentration of pyridine nucleotides in the liver, indicates either the absence of an exchange DPN-ase, or its negligible effect on the metabolism of INH.

The results clearly indicate that a relationship exists between the toxicity of INH and pyridoxine. The nature of the competitive action, however, is not obvious. While the growth depression observed in rats on a pyridoxine deficient ration supplemented with INH was striking, other symptoms of B_6 deficiency were lacking. Other studies showed that large amounts of XA were excreted by rats receiving desoxypyridoxine for 1 week, whereas animals given INH excreted less than the control rats. Thus the deleterious effects obtained with INH are not related to some of the symptoms heretofore considered characteristic of pyridoxine deficiency in the rat. Biehl and Vilter(2) suggested . . . "either a disorder in renal conservation of B_6, or a chemical alteration of this vitamin by INH and its excretion" as an explanation of their results. Our data indicate that pyridoxine, or more likely pyridoxal, functions as a detoxifying agent for INH.

Yoneda *et al.*(10,11) have demonstrated that colon bacillus tryptophanase and arginine decarboxylase, both of which require pyridoxal phosphate as a coenzyme, are inhibited by INH. The inhibition of *M. tuberculosis* H 37 RV by INH has been reported to be reversed by pyridoxal(12); however, in a similar study with this organism pyridoxine and its derivatives were without effect(13). Although these studies suggest an antagonism, there is as yet no direct evidence that INH exerts its antituberculous activity by inhibiting an enzyme activated by pyridoxal phosphate. Dietrich and Shapiro(14) found that the cysteine desulfhydrase activity of mouse liver was decreased in pyridoxine deprived animals, but that desoxypyridoxine did not lower the concentration of the enzyme in the liver. These data and our observations point to the possibility that a vitamin antagonist might be specific in its action against a single enzyme only, and need not function as an antagonist against all biochemical functions of the vitamin.

Summary. INH did not inhibit any of the normal pathways of niacin metabolism investigated in the rat. When added to a B_6-deficient ration INH had a pronounced toxic effect characterized by inhibition of growth, decreased survival time and epileptiform convulsions. The elevated excretion of xanthurenic acid, considered characteristic of the B_6-deficient rat, was not apparent in INH-treated animals.

1. McConnel, R. B., and Cheetam, H. D., *Lancet,* 1952, v ii, 959.

2. Biehl, J. P., and Vilter, R. W., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 389.

3. Ungar, J., Parkin, K. R., Tomich, E. G., and Muggleton, P. W., *Lancet,* 1954, v ii, 220.

4. Hubbel, R. G., Mendel, L. B., and Wakeman, A. J., *J. Nutr.,* 1937, v14, 273.

5. Robinson, J., Levitas, N., Rosen, F., and Perlz-

weig, W. A., *J. Biol. Chem.*, 1947, v170, 653.
6. Rosen, F., Lowy, R. S., and Sprince, H., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 399.
7. Mann, P. J. G., and Quastel, J. H., *Biochem. J.*, 1941, v35, 502.
8. Zatman, L. J., Kaplan, N. O., Colowick, S. P., and Ciotti, M. M., *J. Biol. Chem.*, 1954, v209, 453.
9. ———, *ibid.*, 1954, v209, 467.
10. Yoneda, M., and Asano, N. A., *Science*, 1953, v117, 277.
11. Yoneda, M., Kato, N., and Okajima, M., *Nature*, 1952, v170, 803.
12. Pope, H., *Am. Rev. Tub.*, 1953, v68, 938.
13. Boone, I. U., and Woodward, K. J., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 292.
14. Dietrich, L. S., and Shapiro, D. M., *ibid.*, 1953, v84, 555.

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Effect of Chronic Ethanol Intoxication on Liver Lipid Content of Rats.* (21553)

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The earlier clinical and experimental evidence indicating a correlation between chronic alcoholism and fatty infiltration of the liver and/or cirrhosis has been amply reviewed (1,2,3). The modern concept that the hepatic lesions found in chronic alcoholics are entirely due to inadequate nutrition, especially insofar as lipotropic substances are concerned, has been questioned recently. Thus, it has been suggested that ethanol *per se* exerts a direct effect, which, if continued over a long enough period of time, leads to fatty infiltration and fibrosis(4,5,6). Other investigators have obtained data that do not support this view(7), or have indicated that the effect of ethanol on liver fat is non-specific, and may be seen when any other source of extra calories, such as sucrose, is fed in conjunction with a diet which is sufficiently low in lipotropic substances(8). In the above mentioned investigations, ethanol was administered to rats as a 10% or 15% solution in place of drinking water(5,6,7,8) or partly so (4). Rats receiving alcohol in this manner are never visibly intoxicated, and exhibit low tissue alcohol-levels.

In the absence of general agreement on the subject of the effect of alcohol on liver lipid content, the problem was re-examined in rats fed by stomach tube. By this technic it was possible to accomplish three ends: 1) the alcoholic animals were subjected periodically to intoxicating doses of alcohol and thereby experienced high tissue alcohol-levels; 2) they were forced to consume an adequate diet at the same time; and 3) it was possible to pair-feed alcoholic rats and control rats with great precision. The results of the experiments performed under these conditions demonstrate that ethanol, in contrast to caloric-ally equivalent quantities of carbohydrate, promotes the accumulation of liver lipids within a short period of time, even when the diet is adequate with respect to lipotropic substances for the non-alcoholic control animals.

Procedure. Male albino rats (approximately 185 g weight at beginning of experimental period) were fed entirely by stomach tube for about 30 days. Food was administered as synthetic rations suspended in water. Additional water was given *ad libitum*. Each animal received 2 feedings per day. Prior to experimental period, the rats were subjected to a one week period of adaptation to stomach-tube feeding, after which they were able to receive between 9 and 11 ml of suspended ration per feeding. Animals in each alcoholic group received the same diet, quali-

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tatively and quantitatively, as did animals in the corresponding control group, with one exception: the second meal of the alcoholic rats contained ethanol, isocalorically substituted for an equivalent quantity of carbohydrate present in the control diet. Ethanol was assumed to supply 7 calories/g. Ethanol was mixed with the dry dietary constituents and diluted with water immediately prior to feeding, thus avoiding any loss due to evaporation. Total exposure to air of each alcohol-diet mixture, in which the alcohol was present as a 21% to 25% solution by volume, did not exceed 10 minutes. Non-alcoholic diets were also kept as solid powders and diluted with water immediately before feeding, inasmuch as a newly formed suspension was found to have maximum fluidity. Each rat received a total of 48 calories per day, except in one experiment with somewhat older rats, in which each animal received 60 calories per day. Each feeding provided half the total daily caloric intake. The alcoholic animals obtained 22% of their total daily caloric intake as ethanol (1.52 g ethanol/rat/day for 48 cal. rats, and 1.9 g ethanol/rat/day for 60 cal. rats). Ethanol administered was sufficient to render animals quite intoxicated for about 12 hours. The *diet* was adequate for maintenance of good growth and normal liver lipid values in control groups, although the daily caloric intake of rats was somewhat lower than would have been the case had they been allowed to eat *ad libitum*. Limitation on total food intake was imposed by the quantity of diet that could be fed by stomach tube at one time. The synthetic basal diet was composed of the following: carbohydrate 77%; mazola oil, 5.0%; vitamin-test casein, 14.0%; and Phillips and Hart salt mix, 4.0%. To each 100 g of diet were added the following vitamins: choline chloride, 25.0 mg; p-amino benzoic acid, 20.0 mg; inositol, 50.0 mg; thiamine hydrochloride, 0.4 mg; riboflavin, 0.6 mg; niacin, 1.0 mg; pyridoxine, 0.4 mg; calcium pantothenate, 1 mg; biotin, 0.4 mg; menadione, 0.5 mg; mixed tocopherols, 12 mg; vit. A, 1260 U.S.P. units, vit. D, 126 U.S.P. units. At the *end of experimental periods*, the rats were sacrificed by decapitation, the livers quickly removed, and analyzed for total lipid according to a modification of the method of Payne(9).

Results. Liver lipids. In every experiment in which the basal diet was employed, the rats chronically intoxicated with alcohol accumulated liver lipids in excess of the amount found in the corresponding isocalorically fed controls (7.1-7.6% versus 5.3-5.7% on a fresh tissue weight basis). All differences between alcoholics and controls were statistically significant ($P<0.01$). The same results were obtained with diets containing glucose, sucrose, or half sucrose and half corn starch. A preliminary experiment with 21 normal rats maintained on Purina Chow pellets had shown that normal liver lipid concentrations ranged from 5.0% to 5.7%, averaging 5.4%, according to our method of lipid analysis. Thus, within the comparatively short experimental period of one month, the alcoholic animals had increased their liver lipid concentrations to values about 36% above normal. Dry weight determinations showed that there were no differences in liver water content between the alcoholic and the control rats. The results are summarized and illustrated in Fig. 1.

Lipotropic agents. Since it had been shown that supplementation of diets low in lipotropic substances with choline, methionine, or casein tends to abolish the effects of ethanol on the liver fat(6,8), and since it had been postulated that ethanol increases the choline requirement of an animal(5,10), it was pertinent to inquire whether the lipotropic vita-

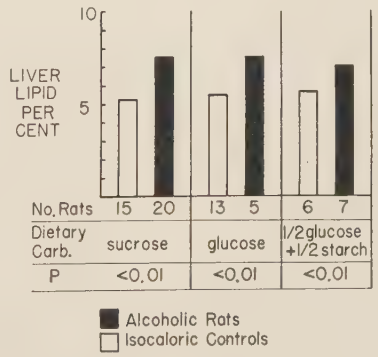

FIG. 1. Liver lipid concentrations (as % of fresh tissue), of chronically intoxicated rats and their isocalorically-fed, non-alcoholic controls, on same diet.

mins would prevent the accumulation of liver lipid under the conditions of these experiments. It was found that, while supplementation with ordinary quantities of either choline chloride or vit. B_{12} did not prevent the accumulation of liver lipids in the alcoholics, the addition of relatively large quantities of these substances completely prevented fatty infiltration. Thus alcoholic rats receiving supplements of 1 mg and 4 mg of choline chloride/rat/day, or 2.9 μg of vit. B_{12}/rat/day still showed high liver lipid values in comparison with the corresponding supplemented, non-alcoholic controls ($7.3 \pm 0.46\%$, $7.0 \pm 0.34\%$ and $6.2 \pm 0.87\%$, versus $5.1 \pm 0.16\%$, $5.0 \pm 0.15\%$ and $5.2 \pm 0.33\%$ respectively). Alcoholic rats receiving supplements of 50 mg choline chloride/rat/day or 6.8 μg vit. B_{12}/rat/day however, had completely normal liver lipid concentrations; thus, alcoholics, $5.4 \pm 0.09\%$ and $5.0 \pm 0.22\%$; controls, $5.3 \pm 0.13\%$ and $5.1 \pm 0.08\%$.

Growth rate. The effect of alcohol on growth has also been a subject of controversy (11,12), most of the investigations concerned with this problem not having provided for a rigidly controlled dietary situation. In our experiments it was observed that the chronically intoxicated rats grew at considerably lower rates than did the control animals on the same diet and receiving the same caloric intake, despite the fact that the physical activity of the alcoholic rats was much less than that of the controls, particularly during the periods of intoxication. Thus the groups receiving ethanol gained only 34% to 75% as much weight as did their corresponding controls during the experimental period. The reasons for this discrepancy in growth rates are not apparent. Typical growth curves are illustrated in Fig. 2.

Discussion. Our results show that ethanol, when given in high doses, promotes the accumulation of liver lipid in the rat within a short period of time. This occurs even when the diet is adequate, as judged by its effects on liver lipid content and growth rates of control animals. Thus, our data do not support the contention(8) that alcohol may promote fatty infiltration of the liver simply by supplying excess calories, and thereby creating, under certain dietary conditions, an imbalance between total caloric and lipotropic intake. They do suggest that alcohol *per se* has an effect on the liver, directly or indirectly. Since long-standing fatty liver may ultimately lead to fibrosis and cirrhosis, this effect of alcohol on the liver may be a factor in the development of liver lesions in alcoholics. The specific mechanism by which ethanol exerts its effect on the liver, and the precise manner in which the lipotropic agents may be involved, are as yet unknown.

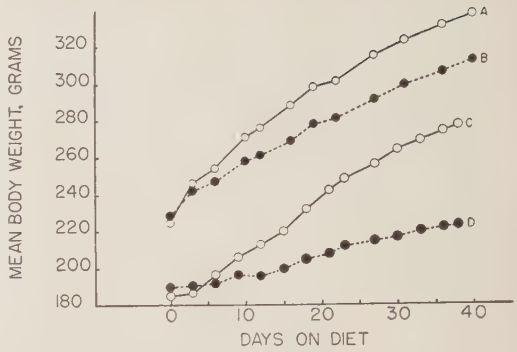

FIG. 2. Typical growth curves of chronically intoxicated rats and their isocalorically-fed, non-alcoholic controls, on same diet. Exp. I, each rat receiving 60 cals/day: A = controls, B = alcoholics. Exp. II, each rat receiving 48 cals/day: C = controls, D = alcoholics.

Our data also clearly indicate that ethanol does not support growth as well as do isocaloric quantities of carbohydrate, even when all other dietary conditions are exactly alike. The differences in the observed growth rates cannot be explained by the small losses of alcohol due to excretion via the lungs and kidneys. Heat loss as a result of vascular dilatation in the skin of the alcoholic rats may be a factor, although precautions were taken to keep the rat room warm at all times (75-80°C). Differences in absorption may also be a factor, although the alcoholic rats did not show any diarrhoea. The mechanism for this growth inhibiting effect of ethanol has not been elucidated.

Summary. 1. Chronic alcoholic intoxication was produced in rats by tube feeding diets in which ethanol was isocalorically substituted for some of the carbohydrate in the control diet. The intoxicated rats uniformly

showed an increase of about 36% in liver fat within approximately 30 days. This occurred on diets that were adequate for maintenance of good growth and normal liver lipid concentrations in non-alcoholic animals. 2. Large supplements of the lipotropic vit. B_{12} and choline, protected against this fatty infiltration of the liver. 3. Chronically intoxicated animals grew at a significantly lower rate than did isocalorically fed non-alcoholic controls on the same diet.

1. Moon, V. H., *Arch. Path.*, 1934, v18, 381.
2. Jolliffe, N., and Jellinek, E. M., *Quart. J. Stud. Alc.*, 1941, v2, 544.
3. Klatskin, G., *Yale J. Biol. and Med.*, 1953, v26, 23.
4. Ashworth, C. T., PROC. SOC. EXP. BIOL. AND MED., 1947, v66, 382.
5. Klatskin, G., Gewin, H. M., and Krehl, W. A., *Yale J. Biol. Med.*, 1951, v23, 317.
6. Klatskin, G., Krehl, W. A., and Conn, H. O., *J. Exp. Med.*, 1954, v100, 605.
7. Forbes, J. C., and Duncan, G. M., *Quart. J. Stud. Alc.*, 1950, v11, 373.
8. Best, C. H., Hartroft, W. S., Lucas, C. C., and Ridout, J. H., *Brit. Med. J.*, 1949, v2, 1001.
9. Payne, R. W., *Endocrinology*, 1949, v45, 305.
10. Klatskin, and Krehl, W. A., *J. Exp. Med.*, 1954, v100, 615.
11. Mitchell, H. H., *J. Nutr.*, 1935, v10, 311.
12. Mitchell, H. H., and Curzon, E. G., *Quant. J. Stud. Alc.*, 1940, v1, 227.

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Electrolytes and Water in Muscles of Rat Forelimb After Intense Local X Irradiation.* (21554)

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Investigations have been made for the purpose of determining whether, in muscle fibers, intense local X irradiation will cause an exchange of outside Na for inside K as it did in erythrocytes for Sheppard and Beyl(1). The literature found is based mostly on total-body-irradiation experiments done for other purposes, wherein the doses used are too low to influence refractory muscle cells. For whole-body exposure at these low doses(2,3) single tissues are influenced secondarily by effects imposed elsewhere in the body. Dehydration may occur because of diuresis, diarrhea, or low food intake. There may be shifting of extracellular fluid within the body as from muscle to the very sensitive gut. Other effects may occur secondary to endocrine disturbance. We have avoided these difficulties by exposing only a small mass of muscle in the forelimb of a rat to large doses of X rays while the remainder of the body is shielded.

Too often, the results of electrolyte analyses are expressed in terms of wet weight, so that water shifts dilute out the true changes. Beutel and Winter(4) did refer to dry weight but they did not estimate the extracellular water phase. Muscle gained water and chloride but it remained unknown whether these changes were cellular or extracellular. We have referred to the more reliable dry weight and have estimated extracellular water as inulin space. In rats not specially subjected to vigorous postirradiation exercise, doses up to 58 kr left water and K of muscle fiber unchanged but caused marked extracellular edema as an example of local physiological disturbance resulting from X-ray exposure.

Methods. Wistar male rats (380 g) anesthetized with intraperitoneal Na pentothal

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† Research Participant, 1951, Oak Ridge Institute of Nuclear Studies, working in Biology Division, Oak Ridge National Laboratory.

TABLE I. Effect of X-Ray Exposure on Distribution of Water, Inulin, and Na^{24} in Rat Muscle. (Data from Series B.)

		g of water/250 g dry wt of muscle (~1 kg fresh normal muscle)						Linear regression parameters from analysis of (X-S) values (see text)	
Dose (kr)→		0.0	36.9	41.0	45.0	65.0	73.4	Intercept *a*	Slope *b*
Total water	S*	700	705	716	730	722	723	63.6	1.332
	X†	735	836	816	850	875	885		
Inulin space	S	117	99	100	122	113	80	95.17	.525
	X	101	214	223	239	222	229		
Na^{24} space	S	169	158	168	186	177	152	52.83	1.469
	X	146	270	275	310	310	324		
Cell water	S	583	606	616	608	609	643	−31.57	.807
	X	634	622	593	611	653	656		

* Shielded muscle. † X-irradiated muscle.

(4 mg/100 g of body weight) were secured on an animal board under a 13-mm lead plate shield containing a window 2 cm in diameter under which either the right or left forelimb alone could be immobilized in a groove for steady exposure to X rays. Muscles of the opposite, shielded, forelimb served as controls. X rays were supplied by a G. E. Maxitron 250 unit (250 kvp, 30 ma, 3 mm of Al filtration hvl about 0.5 mm of Cu) at a rate of 720 r/min as measured with a 100-r Victoreen r meter. All exposures were 30 cm from the shutter. Single exposures were employed, the dose being regulated by the duration of exposure. Scatter beneath the shield averaged 0.11% of the dose as measured with a Victoreen r meter placed at points inside a fresh rat carcass. For example, the chest received 1.6 r/min, the adrenals 0.8 r/min, and the other forelimb 0.7 r/min. For our highest exposure of 73 kr applied to the forelimb, this would be 77 r of scattered radiation impinging upon other tissues. Following X irradiation the animals were kept singly in small cages, where they remained quiescent. In *series A,* water and K were evaluated in muscle taken from rats placed under intraperitoneal Na pentothal (4 mg/100 g of rat) 22 hr after the X ray was applied. Blood was taken from the abdominal aorta into heparin, and the front legs quickly amputated to prevent loss of electrolytes because of the ischemia of hemorrhage. All the muscle between carpus and elbow was removed from each forelimb and cleared of fat, blood, and membranous and tendinous connective tissue. The entire amount of muscle from each leg was minced and mixed together before drying at 105°C for determination of water content and dry weight. The dry material was liquefied in concentrated nitric acid preparatory to the measurement of potassium in the Perkin-Elmer flame photometer(5). In *series B,* total water and inulin and Na^{24} spaces were determined in muscle. Two hours before the muscle was scheduled to be excised the rat was etherized, nephrectomized by retroperitoneal approach, and given, by femoral vein, 13.5 ml/kg of body weight Difco inulin (65 mg/ml) dissolved in 0.15 M NaCl tagged with Na^{24}(0.3 mc/ml). The rat was allowed to recover from the ether; later pentothal was given for the muscle sampling as in series A. All the muscle from one forelimb, about 0.7 mg, was finely minced, mixed together, and weighed wet. Na^{24} in the untreated muscle mince was determined directly in a plastic tube placed inside a 100% geometry gamma-ray ion chamber(6). The Na^{24} space was computed as activity in 250 g of dry muscle divided by activity per liter of blood plasma (not plasma water). Half of this mince was dried at 105°C in an oven to estimate dry weight. The other half was carried through the inulin method of Ross and Mokotoff(7). Dilute alkali liquefies the muscle and autoxidizes any glucose. The color reaction for inulin quantitation is developed by resorcinol-thiourea in a zinc hydroxide filtrate obtained from the neutralized digest. It was read at

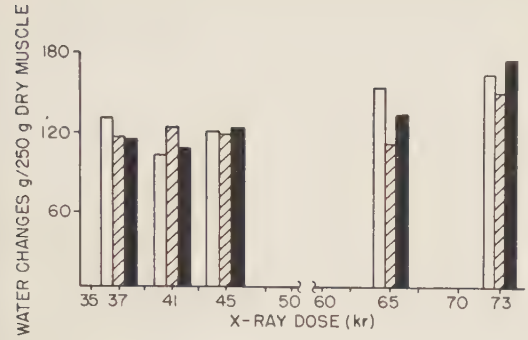

FIG. 1. Influence of X rays on total water content, inulin, and Na^{24} spaces of rat muscle. Changes in total water content between companion shielded and X-rayed muscles (□); changes in inulin spaces (▨) or in Na^{24} spaces (■) between companion shielded and X-rayed muscles are shown. Each set of three columns belongs to a single rat given the dose shown below the columns.

the 520 mμ setting of a Coleman Junior Spectrophotometer. The large dose of inulin given (0.5-0.7 mg of inulin/g of muscle) obviated the need for an inulinoid blank which, in preliminary runs, contributed about 5% to the muscle and plasma readings and was unchanged by X irradiation. The inulin space was computed as inulin per 250 g dry weight of muscle divided by the number of milligrams of inulin per liter of plasma.

Results. During the 22-hr postirradiation period the irradiated limb was not favored in walking when the animal was stimulated into activity, and was capable of prehensile contractions of the toes when the animal was lifted by the tail. No spastic twitchings occurred such as are seen after high-level whole-body exposures. The skin was reddened externally and the forelimb obviously swollen. Neither the isolated muscles nor the subcutaneous tissue contained any observable gross petechial hemorrhages but gelatinous swelling was obvious in both in relation to dosage.

Total water and inulin and Na^{24} spaces are shown in Table I. The differences (X-S) between X-rayed and control limbs are shown in Fig. 1. There is a pronounced radiation effect in which large relative changes occur in total water. These changes are closely paralleled by changes both in inulin and Na^{24} spaces, indicating that the water increases are predominantly extracellular. The changes seem to be of the threshold type, setting in between 10 and 36.9 kr.

Finer aspects of the relation between the changes and the dose were analyzed statistically by fitting linear regression curves to the (X-S) values versus dose for total water, and inulin and Na^{24} spaces. A fit was also made for cell water, taken as total water minus inulin space. The intercepts a and slopes b of the regression lines are given in the last two columns of Table I. For each regression fit, the value of the standard deviation for the individual (X-S)'s was obtained. For the first 3 fits; namely, total water, inulin, and Na^{24}, the standard deviations expressed as percentages of the mean of the individual observations of the set are in each case 11.3, 12, 3, and 9.5%, respectively. Thus the first 3 physiological indices are about equally accurate as objective numerical measures of radiation effect in our experiments. Furthermore, insofar as measurements of changes in space are concerned, the inulin and Na^{24} are about equally satisfactory.

Since at the higher doses an upward trend was recognized in the (X-S)'s for all three physiological indices, the slopes b were subjected to standard variance ratio tests, which detected no significant differences among them. It was thus permissible to lump together the (X-S)'s for the three situations and make a fit to the combined data, yielding a slope of 1.108. This differs significantly from zero at the 0.3% level so that there is a definite tendency for the effect to increase with dose, the effect being imposed upon the threshold character of the dose-effect relation. Both the total water and Na^{24} data increase significantly with dose at the 1.5 and 1.0% levels, respectively. Little confidence can be assigned to the apparent increase in inulin space since the level of significance is greater than 25%.

Because the (X-S)'s for inulin are considerably lower at the two high-dose points, the slope for inulin was compared with that from a regression curve for the total water and Na^{24} spaces combined. The two slopes differ significantly at the 15% level, which is so high that the difference cannot be considered as very significant.

TABLE II. Effect of X Rays on Potassium Content of Rat Muscle (Data from Series A).

	Dose (kr)→	10	29	29	58
K content (meq/g dry wt)	S*	113.7	115.3‡	111.7	117.0
	X†	112.7	117.4‡	111.6	117.0

* Shielded muscle.
† X-irradiated muscle.
‡ 68 hr after irradiation.

It is clear from the tissue analyses for series A (Table II) that the intracellular K is untouched by radiation up to 58 kr. This holds only when K is expressed per unit dry weight since the extracellular water gain would otherwise dilute the calculated K content. Intracellular water differences (X-S) were fitted from the data in the last line of Table I, and the *b* and *a* values are shown in the table. Neither differs from zero at a level of significance less than 25%. It is thus clear that changes in fiber water are not observable in our experiments. If we accept the greater changes in Na^{24} space than in inulin space for the higher doses, a questionable matter to be sure, the result would indicate a small increase in fiber Na.

Discussion. Our observation seems to be the first quantitative physiological recognition by inulin space determinations that the tissue swelling caused by intense local X irradiation may be entirely an accumulation of extracellular fluid, not necessarily including the swelling of tissue cells. In our experiments the threshold for this is below 40 kr. This is lower than the threshold for minimal perceptible damage to the visualized circulation of the bat wing as viewed by Smith, Svihla, and Patt(8). They found that, at doses of 50 and 100 kr, the first visual sign to appear was a sticking of leukocytes onto the walls of small blood vessels in the wing. Only at 200 kr did the leukocytes plug the vessels to cause stagnation such as might induce edema by increasing the hydrostatic pressure in capillaries. In our rats edema appeared at doses less than would be expected to cause stagnation. If edema is not due to stagnation, an accumulation of osmotically active material in the extracellular spaces of the muscle must have drawn the fluid out of the blood vessels. Whether in our experiments the effect arises from leaky capillaries or from the dissolution of tissue cells or connective tissue matrix material is not known, but it may be significant for our results that, at 30 kr in the rabbit, intense necrotic changes have been observed (9). The effect is, of course, entirely local since other areas were effectively shielded.

It should be emphasized that our observations were of animals whose irradiated muscles were not subjected to vigorous exercise following irradiation. There is some evidence that radiation effects appear more rapidly and at lower doses in muscle subjected to a post-irradiation work load. Blood from the coronary sinus of a beating heart in a dog with open chest and under artificial respiration exhibited K elevation after only 12 kr had been applied directly to the heart. Surviving perfused rabbit hearts also lose K during X irradiation(10). X irradiation accelerates the fatiguing process in skeletal muscle at doses as low as 50 kr in excised frog muscle and 72 kr in live rabbit muscle(11). The results were similar in most respects to those occurring when the synthesis of energy-rich phosphate is interfered with, and suggest an interference of some sort with the biochemical energy sources.

According to a current concept, Na moves out of muscle fibers through an energetic transfer mechanism with K entering passively to replace the Na(12). The action of agents which can impair Na transport, such as radiation, often becomes detectable only when Na transport is overloaded. For instance, cardiac glycoside preparations noticeably affect Na and K movements in skeletal muscle only when the Na extrusion mechanism is overworked, as when muscle is immersed in low K media(13). Contractile activity induces release of muscle K and uptake of Na(14). It is thus conceivable that impairment of the Na extrusion mechanism by X irradiation at 65-73 kr might be demonstrated as a Na gain or K loss in muscle subjected to postirradiation work load.

Summary. Intense radiation up to 73 kr was applied locally to the forelimb muscles in a rat with its body otherwise shielded. Observations were confined to limbs not subjected to active fatigue following X-ray exposure. For up to 22 hr after exposure the

muscles remain functional. However, the radiation produces marked extracellular edema as measured by inulin space and Na^{24} space determinations. The muscle fibers lose no K per unit dry weight but may possibly gain minimal Na as Na^{24}.

1. Sheppard, C. W., and Beyl, G. E., *J. Gen. Physiol.*, 1951, v34, 691.

2. Painter, E. E., and Cooley, M. A., *Fed. Proc.*, 1951, v10, 100.

3. Patt, H. M., *Ann. Rev. Physiol.*, 1954, v16, 51.

4. Beutel, A., and Winter, K. A., *Strahlentherapie*, 1935, v54, 89.

5. Walker, W. G., and Wilde, W. S., *Am. J. Physiol.*, 1952, v170, 401.

6. Jones, J. W., and Overman, R. T., A.E.C. Declassified Document AECD 2367, Office of Technical Services, Dept. of Commerce, Washington, D.C.

7. Ross, G., and Mokotoff, R., *J. Biol. Chem.*, 1951, v190, 659.

8. Smith, D. E., Svihla, G., and Patt, H. M., *Physiol. Zool.*, 1951, v24, 249.

9. Milch, L. J., *et al.*, *Science*, 1954, v120, 713.

10. Ellinwood, L. E., and Coon, J. M., private communication.

11. Gerstner, H. B., Lewis, R. B., and Richey, E. O., *J. Gen. Physiol.*, 1954, v37, 445.

12. Wilde, W. S., *Ann. Rev. Physiol.*, 1955, v17, in press.

13. Matchett, P. A., and Johnson, J. A., *Fed. Proc.*, 1954, v13, 384.

14. Fenn, W. O., and Cobb, D. M., *Am. J. Physiol*, 1936, v115, 345.

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Effect of 6063, Sodium Bicarbonate and Ammonium Chloride on Electrolyte Composition of Thermal Sweat.* (21555)

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(Introduced by John P. Peters.)

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Whereas the excretory and thermoregulatory functions of sweat glands have been extensively studied(1,2,3) their conservative functions or their role in the preservation of volume and composition of body fluids have received relatively minor attention(4,5,6). Sweat glands are capable of conserving sodium and chloride when a negative balance of these ions arises from any cause(5) and a fall in rate of thermal sweating probably occurs during dehydration or a rise in osmolarity of body fluids(1,3,7). In these conservative functions cells of sweat glands show a qualitative functional similarity to those of renal tubules.

The present study was undertaken to compare the response of kidney and sweat glands to alterations in the acid-base composition of body fluids† and to administrations of a powerful carbonic anhydrase inhibitor, 6063.‡

Materials and methods. Four normal males, ages 20-25, were exposed at weekly intervals for 1 to 1½ hours in a room maintained at 120° F.D.B. and 93° to 95° F.W.B. The study consisted of 8 exposures between June 1953 and August 1953. One subject (J.E.) who ingested no special materials prior to any of his exposures, served as control for the group. The other 3 subjects ingested the experimental drug for 2 days prior to, and on the morning of, the collection of sweat. The experiment was so designed that the second

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† Since completion of this study the excellent paper of Amatruda and Welt(7) has appeared. These authors found that alteration of the acid-base balance did not appear to modify the composition of sweat remarkably as compared to modification of the urine.

‡ Generous supplies of carbonic anhydrase inhibitor No. 6063 were made available by the Lederle Co., division of American Cyanamid Co., Stamford, Conn.

TABLE I. Experimental Design.

Subject	Exposures 1	2	3	4	5	6	7	8
T.M.	Control	6063	$NAHCO_3$	NH_4Cl	NH_4Cl	$NAHCO_3$	6063	Control
L.S.	NH_4Cl	$NAHCO_3$	6063	Control	Control	6063	$NAHCO_3$	NH_4Cl
J.E.	Control	Control	Control	"	"	Control	Control	Control
A.M.	6063	NH_4Cl	"	$NAHCO_3$	$NAHCO_3$	"	NH_4Cl	6063

Dose of drugs as follows: (1) 6063—1 g daily in 4 divided doses and 250 mg on morning of collection; (2) NH_4Cl—15 g daily (5 divided doses), 5 g the morning of collection; (3) $NAHCO_3$—20 g daily (5 divided doses), 5 g the morning of collection.

4 exposures to heat reversed the order of the first 4 of the series, and the order of succession was different in each of the 3 subjects. This is illustrated in Table I. This design was utilized to control the effect of repeated exposures to heat, acclimatization during the summer months, and any alteration in the composition of sweat produced by the drug administered during the *previous* exposure. Arms and hands of each subject were scrubbed thoroughly with soap and water, rinsed with distilled water, and then washed with ethyl ether§ prior to entering the hot room. No *sweat collections* were made during the first 15 minutes of sweating. After this "flushing" period, the importance of which has been previously described(8), the arms and hands were rinsed thoroughly with distilled water, dried, and encased in plastic bags. The latter were sealed at the level of the insertion of the deltoid muscle with a strip of firm elastic 3 inches in width. Each subject was weighed to the nearest $\pm$ 10 g before and after period of sweat collection. While sweat was being collected the subjects moved their arms lightly, and at 10-15 minute intervals the plastic bags were patted against the skin of the arms. This procedure ensured mixing of sweat on the skin with the more dilute water vapor on the walls of the bag. After the period of collection the bags were quickly removed, vigorously shaken and the sweat was poured under a one-half inch layer of oil in a plastic container. Samples were immediately taken for the determination of ammonia(9), titratable acidity(10), pH,‖ total CO_2 content(11), and lactate(9). The sweat was then filtered and analyzed for sodium, potassium and chloride by methods previously described(8). *Urine* was collected under oil from each subject for the 24 hour period preceding exposure to heat. Toluene was used as a urinary preservative. A sample of venous blood was drawn under oil in a heparinized syringe the morning of sweat collection. Urine was analyzed for everything measured in sweat with the exception of lactate, blood for everything except lactate, ammonia and titratable acid.

Results. In all subjects the qualitative changes in urine and blood following 6063, ammonium chloride, and sodium bicarbonate were the same, and typical of those previously described(12,13,14) after administration of these substances. Fig. 1 illustrates these changes in one subject (A.M.)

Concentrations of the various solutes in sweat collected in these experiments, reflect the site and mode of collection. The values are not necessarily comparable to those of

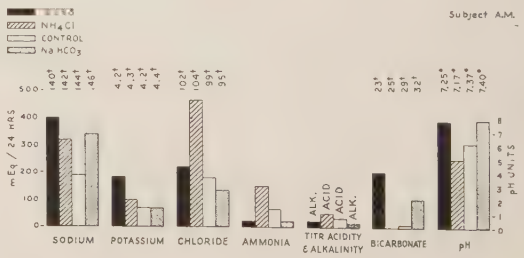

FIG. 1. Changes in composition of urine and serum following 6063, ammonium chloride, and sodium bicarbonate.

* pH of blood.

† Concentrations of various solutes in blood in mEq/liter.

§ The wash with ether was used to remove the fatty acids and acidic oils from the skin surface. This is essential if the pH of the sweat is to be measured.

‖ A Beckman pH meter with an anaerobic electrode assembly was used for all pH determinations.

TABLE II. The Composition of Thermal Sweat* following Ingestion of NH_4Cl, 6063, and $NAHCO_3$.

Date (1953)	Subject	Drug	pH	Titratable acid, mEq/l	Ammonia, mEq/l	Sodium, mEq/l	Potassium, mEq/l	Chloride, mEq/l	Lactate, mEq/l
6/15	T.M.	Control	5.6	2.6	4.0	46.2	6.1	39.0	—
23		6063	5.3	2.8	5.5	29.6	6.2	19.1	—
30		$NAHCO_3$	5.6	3.5	5.8	29.4	5.9	16.5	—
7/ 3		NH_4Cl	5.8	2.0	3.2	35.3	4.7	28.0	4.5
7		"	5.5	2.0	4.4	—	—	19.7	15.8
14		$NAHCO_3$	6.5	1.4	3.0	54.4	4.2	44.5	8.2
21		6063	5.4	2.6	4.7	39.8	6.1	32.9	8.8
27		Control	5.8	2.0	3.0	44.0	5.3	40.8	8.4
6/15	A.M.	6063	5.9	1.5	3.6	38.8	5.0	31.8	7.5
23		NH_4Cl	5.1	2.0	3.5	38.8	6.8	29.3	5.9
30		Control	5.2	3.0	4.1	36.8	6.1	26.1	17.4
7/ 3		$NAHCO_3$	6.0	2.0	2.7	35.6	3.8	25.7	4.2
7		"	5.4	1.4	3.4	30.4	4.1	23.1	5.7
14		Control	5.8	1.8	3.2	41.3	6.4	34.1	2.0
21		NH_4Cl	5.1	2.2	3.7	18.7	5.6	21.0	2.2
27		6063	5.1	3.0	3.2	27.4	5.5	24.8	6.0
6/15	L.S.	NH_4Cl	6.0	0.9	3.3	82.4	5.5	77.6	6.5
23		$NAHCO_3$	5.5	2.0	3.4	67.0	5.1	55.7	12.5
30		6063	5.2	3.4	3.7	45.1	4.0	38.8	4.9
7/ 3		Control	5.0	4.0	2.2	34.9	4.3	24.6	7.0
7		"	5.3	1.6	2.5	53.2	4.2	52.2	8.2
14		6063	6.0	1.0	2.9	72.7	4.2	62.9	4.2
21		$NAHCO_3$	5.6	2.6	3.0	52.4	4.2	47.1	3.8
27		NH_4Cl	5.0	2.4	3.0	51.7	5.3	48.9	5.4
6/15	J.E.	Control	5.6	3.6	3.8	75.7	6.0	61.2	6.4
23		"	5.0	3.4	4.3	64.7	6.3	55.5	15.8
30		"	5.0	3.0	4.0	51.6	4.5	44.1	12.6
7/ 3		"	4.7	4.6	3.8	49.4	5.8	41.1	8.4
7		"	4.7	4.0	3.5	51.4	5.6	45.6	9.4
14		"	5.4	2.2	3.7	77.4	5.5	64.0	3.9
21		"	5.0	3.2	4.5	65.1	5.7	54.9	5.0
27		"	4.9	3.6	3.7	43.5	5.7	39.5	11.5

* There was no measurable quantity of CO_2 in the sweat.

sweat collected from the surface of the entire nude body(8). However the standardized procedure used allowed careful comparison of the effects of each drug on composition of sweat. The latter is presented in tabular form in Table II. As each subject showed surprisingly little variation in sweat rate, this variable has not been presented.

It is apparent from Table II that there is no consistent relationship between the acid-base balance of body fluids and the ammonia, titratable acidity, pH or lactic acid of sweat. This is contrasted with the marked changes in urinary composition and pH following ingestion of 6063, ammonium chloride and sodium bicarbonate (Fig. 1). Concentration of potassium in sweat, approximated the values in normal serum, varied greatly from subject to subject, and bore no relationship to the substance ingested. Concentrations of sodium and chloride consistently decreased during the first four exposures to heat in all subjects. This probably represented an acclimatization phenomenon(3,4) rather than any effect of the substances ingested. The change in concentration of sodium and chloride during the last four exposures was not consistent.

To confirm statistically the observations described above the data of Table II were analyzed by a simple ranking procedure.¶ This statistical approach confirmed the interpretation derived from simple observation of the data.

Discussion. Under conditions of the present study, collected sweat was a moderately acid secretion, low in ammonia and titratable acid and of poor buffering capacity. Sweat maintained these characteristics regardless of the substance ingested or the alteration in acid-base balance produced. From the nega-

tive effect of 6063 it is apparent that in contrast to urine the content of hydrogen ion in thermal sweat is not dependent on carbonic anhydrase activity. It probably results primarily from the content of lactic acid. The latter's dissociation constant of 1.38×10^{-4} and the concentrations found adequately account for the observed concentrations of hydrogen ion. Weiner and von Heyningen(15) have shown that concentration of lactate in thermal sweat has no consistent relationship to its concentration in extracellular fluids. The evidence to date(15-17) strongly suggests that lactic acid of thermal sweat is derived from the metabolism of the sweat gland itself. Any bicarbonate ion which may enter the sweat from the extracellular fluid is rapidly neutralized by lactic acid.

Under the conditions of collection used it is very unlikely that the low levels of ammonia (2.7 to 5.8 mEq/l) were due to bacterial or non-bacterial decomposition of the urea in thermal sweat. The values of ammonia in this study were very similar to those of Amatruda and Welt(7), Mezincesco(18) and Itoh and Nakayama(19). The latter investigators have rather conclusively demonstrated that the ammonia in sweat is not secreted by the sweat gland itself but results primarily from deamination of glutamine by skin glutaminases. Sweat proper has no glutaminase activity(19).

In contrast to the kidney, the sweat gland has no regulatory role in the maintenance of the hydrogen ion concentration of the body fluids. However, in its ability to conserve sodium and chloride it holds a position between the gastrointestinal tract and the kidney. The former has no conservative function.

Summary. 1. Thermal sweat, as collected in this study is a moderately acid secretion low in ammonia and titratable acid. 2. Ingestion of a carbonic anhydrase inhibitor (6063), sodium bicarbonate and ammonium chloride had no effect on the acid-base composition of sweat regardless of changes in composition of extracellular fluid and urine. 3. Sweat glands have no regulatory role in the maintenance of hydrogen ion concentration of body fluids.

¶ This statistical approach was used because in setting up the experiment the effect of treatment was not completely separated from the effect of subject. Effects of treatment were examined by taking results for each of 8 separate days and considering values obtained for any particular variable on each day. These values for any one day were ranked in order (1 to 3) and an inspection made to find whether any particular treatment obtained consistently high or consistently low ranks on each day. Similarly, ignoring the effect of days, the effect of the 3 forms of treatment on any variable were ranked against each subject. The authors are indebted to Dr. C. White of the Biometric section of the Department of Public Health, Yale University for his suggestions on the statistical analysis of the data.

1. Kuno, Y., *The Physiology of Human Perspiration.* London, Churchill, 1934.

2. Lobitz, W. C., Jr., and Mason, H. L., *Arch. Dermat. and Syph.,* 1948, v57, 907.

3. Robinson, S., Gerking, S. D., Turrell, E. S., and Kincaid, R. K., *J. Appl. Physiol.,* 1950, v2, 654.

4. Conn, J. W., Advances in Internal Medicine III. New York, *Interscience,* 1949, p373.

5. McCance, R. A., *J. Physiol.,* 1938, v92, 208.

6. Ladell, W. S. S., *Brit. M. Bull.,* 1945, v3, 175.

7. Amatruda, T. T., Jr., and Welt, L. G., *J. Appl. Physiol.,* 1953, v5, 759.

8. Kleeman, C. R., Bass, D. E., and Quinn, M., *J. Clin. Invest.,* 1953, v32, 736.

9. Conway, E. J., *Microdiffusion Analysis and Volumetric Error.* London, Lockwood, 1947.

10. Peters, J. P., and Van Slyke, D. D., *Quantitative Clinical Chemistry* Vol. II. Methods. Baltimore, Williams, and Wilkins, 1932.

11. Hawk, P. B., Oser, B. L., and Summerson, W. H., *Practical Physiological Chemistry,* New York, Blakiston Co., 1947.

12. Pitts, R. F., Ayer, J. L., and Schiess, W. A., *J. Clin. Invest.,* 1949, v28, 35.

13. Pitts, R. F., Lotspeich, W. D., Schiess, W. A., and Ayer, J. L., *ibid.,* 1948, v27, 48.

14. Maren, T. H., Mayer, E., and Wadsworth, B. C., *Bull. J. Hopkins Hosp.,* 1954, v95, 199.

15. Weiner, J. S., and von Heyningen, R., *J. Appl. Physiol.,* 1952, v4, 734.

16. Dill, D. B., Jones, B. F., Edwards, H. T., and Obery, S. A., *J. Biol. Chem.,* 1933, v100, 755.

17. Whitehouse, A. G. R., *Proc. Royal Soc.,* London, series B., 1931, v108, 326.

18. Mezincesco, M. D., *Bull. de la Soc. de Chemie Biol.,* 1938, v20, 39.

19. Itoh, S., and Nakayama, T., *Jap. J. Physiol.,* 1953, v3, 133.

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Enzyme Studies in Muscular Dystrophy. I. Muscle Proteolytic Activity and Vitamin E-Deficiency.* (21556)

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Milman *et al.*(1) have reported that the diaphragm and skeletal muscles of rabbits with muscular dystrophy resulting from vit. E-deficiency release free amino nitrogen more slowly than do similar tissues from normal animals, when the method of Kline(2) is employed. These *in vitro* results suggested that the diminution in muscle mass characteristic of the muscular dystrophy might be due primarily to decreased protein synthesis rather than to an accelerated rate of protein breakdown. In order to investigate further the problem of protein metabolism in this form of muscular dystrophy we have studied the activity of the rabbit muscle proteolytic enzyme system described by Snoke and Neurath(3).

Methods. Young rabbits (900 g) were kept in metabolism cages and given the dystrophy-producing Diet 11 of Goettsch and Pappenheimer(4) which had been treated with 1% ethereal $FeCl_3$. On the basis of previous observations, the onset of muscular dystrophy was assumed to have occurred when the urinary creatine had risen above 35-40 mg per day. Control animals were given Diet 11 supplemented with tocopherol. Animals were sacrificed, after stunning, by exsanguination. Mixed portions of muscle from the back and thighs were homogenized with twice their volume of 2% KCl at pH 6.0 in a Waring Blender. After storing for 18-20 hours the following 3 types of muscle preparation were made from the homogenate: 1) *Low-speed extract.* The muscle homogenate was centrifuged for 20 minutes at 600 x g and the supernatant diluted with an equal volume of 2% KCl before assay. 2) *Whole homogenate.* The muscle homogenate was diluted with 2 volumes of 2% KCl and strained through gauze to permit pipetting. 3) *High speed extract series.* The muscle homogenate was centrifuged for 30 minutes at 15000 x g and the supernatant removed. The residue was resuspended in a volume of 2% KCl equal to that of the previous supernatant. After 30 minutes a second extract was obtained by repeating the centrifugation. Three more 30-minute extracts were obtained by repeating the above procedure. Each extract was assayed individually. The first extract was diluted with an equal volume of 2% KCl before determination of proteolytic activity. All of the above operations were carried out at 0-5°C. Nitrogen content of all of the preparations was determined by a colorimetric nesslerization procedure. The *assay system* was essentially that of Anson(5). To 5 ml of urea-denatured hemoglobin substrate adjusted to pH 4.0 with 1 M acetic acid, were added 5 ml of the muscle preparation and either 5 ml of 0.12 M ferrous ammonium sulfate or 5 ml of water. The mixtures were incubated at 37.5 ± .5°C and 2 ml aliquots were periodically withdrawn and added to 5 ml portions of 5% trichloroacetic acid. The precipitated proteins were removed by centrifugation and the optical density of the supernatants was determined at 280 mμ in a Beckman spectrophotometer. The experimental values were corrected by subtraction of values obtained with blanks that contained all components of the incubation mixtures, except the muscle preparation. In experiments in which hemoglobin was absent, the incubation mixtures contained a urea-acetate buffer solution.

Results. The effect of vit. E-deficiency on the proteolytic activity of the low speed muscle extracts was investigated in 4 series of experiments: 1) in the presence of both hemoglobin substrate and ferrous ion, 2) in the presence of hemoglobin substrate alone, 3) in the presence of ferrous ion alone, 4) in the absence of both hemoglobin and ferrous ion.

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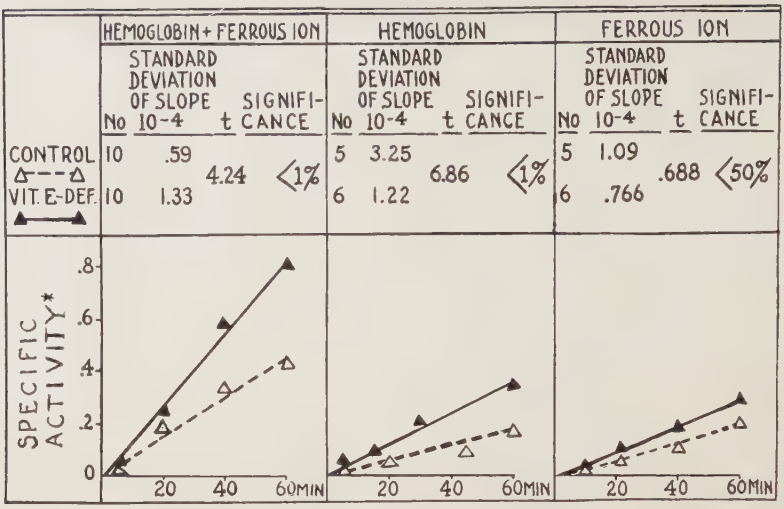

FIG. 1. Effect of Vit. E-deficiency on proteolytic enzyme activity of muscle (low speed extracts).

* Specific activity = change in optical density/mg of muscle protein nitrogen/ml of incubation mixture.

In the absence of both hemoglobin substrate and ferrous ion(4), there was no measurable activity at the end of 60 minutes with the low speed extracts of muscle from either the control or vit. E-deficient rabbits. The results of the other 3 series of experiments are plotted in the figure. Each point represents the average of values obtained from 5 to 10 animals. The curves are calculated by the method of least squares(6). In the presence of ferrous ion and hemoglobin substrate the proteolytic activity of low speed extracts from control animals was the same as that reported by Snoke and Neurath(3) under similar conditions. In systems containing hemoglobin, vit. E-deficiency increased proteolytic activity 80% in the presence of ferrous ion and 110% in the absence of this ion. These differences between the slopes of the activity curves of the control and vit. E-deficient low speed muscle extracts shown in the figure are significant at less than the 1% level. In experiments in which the system contained ferrous ion, but no hemoglobin, vit. E-deficiency increased proteolytic activity 50% but only differences above this level may be considered to be significant. The average protein nitrogen content of the low speed extracts from the control group was 1.56 (0.93-2.72) mg/ml as added to the incubation mixture and 1.51 (1.17-2.67) mg/ml from the deficient group. This small difference in protein nitrogen content as such, could not account for the increased specific activity during the deficiency.

In another group of experiments the effect of vit. E-deficiency on the proteolytic activity of low speed extracts, the high speed extract series, and whole homogenates of muscle was compared. The results reported in the table represent data from 6 control and 5 deficient animals. The deficiency resulted in an increased proteolytic activity with each of the types of muscle preparations used. Due to the decrease in muscle protein nitrogen content during the deficiency, the increase in specific activity of the homogenate was much higher than the increase in activity per g wet weight of muscle. Results of the high speed extract series demonstrate that the proteolytic activity of the homogenate was completely extractable. The first 2 extractions of each high speed series accounted for 75% of the total activity and the proteolytic activity of the fifth extract approached the lower limit of measurement by the procedure used. Proteolytic activity of the individual high speed extracts of the deficient series was higher than

TABLE I. Effect of Vit. E-Deficiency on Proteolytic Enzyme Activity of Muscle.*

	Control	Vit. E-deficient	Δ	% Δ
Low speed extracts				
a) No. of animals	5	5		
Animal numbers	1–3, 5, 6	7–11		
b) mg protein N/ml	1.31 (1.01–1.65)	1.29 (1.12–1.44)		
c) Specific activity†	0.42 (.36–.55)	0.84 (.69–.91)	+0.42	+100
Whole homogenate‡				
d) No. of animals	4	4		
Animal numbers	1, 3–5	7–9, 11		
e) mg protein N/g wet wt muscle	20.4 (15.9–23.3)	14.5 (12.7–15.8)	−5.9	− 29
f) Activity/g wet wt muscle§	4.5 (3.3–6.5)	8.3 (7.0–9.2)	+3.9	+ 87
g) Specific activity, f/e	0.22 (.15–.28)	0.58 (.55–.60)	+0.36	+164
High-speed extract series‖				
h) No. of animals	4	4		
Animal numbers	1, 3–5	7–9, 11		
i) mg protein N extracted/g wet wt muscle	7.53 (6.53–8.51)	7.49 (6.59–7.87)		
j) Activity/g wet wt muscle	4.1 (3.2–4.8)	7.7 (7.3–7.9)	+3.6	+ 88
k) % whole homogenate, j/f × 100	92 (71–119)	92 (84–105)		
l) Specific activity, j/i	0.54 (.43–.69)	1.02 (.95–1.23)	+0.48	+ 89

* Incubation mixtures contained hemoglobin and ferrous ion. Figures in parentheses are the range of values in each group.

† Specific activity = change in optical density/mg muscle protein nitrogen/ml incubation mixture/60 min.

‡ Straining of homogenate to permit pipetting, resulted in loss of muscle material due to retention in gauze. Calculation of homogenate activity based on assumption that material retained was essentially similar to that passed through gauze. Since retention of material by gauze was due to differences in particle size in the homogenate, and activity/g of muscle of high speed extract series was the same as that of homogenate, this assumption appears to be valid.

§ Activity/g wet wt muscle = change in optical density/g wet wt muscle/ml incubation mixture/60 min.

‖ Values obtained by summation of the five individual extracts in each series.

their corresponding controls while protein nitrogen content was the same. Considering that there were a small number of animals in each series and that the different types of muscle preparation were not always obtained from the same animal, the 100% increase in specific activity of the low speed extracts during the deficiency is comparable to and not significantly different from the 89% increase in specific activity of the high speed extract series and the 87% increase of the whole homogenate.

The proteolytic activity of low speed 2% KCl extracts of liver, spleen, and kidney was similarly assayed with hemoglobin substrate and without any added activator. Vit. E-deficiency had no effect on the proteolytic activity of such extracts of any of these tissues.

Discussion. Although the definitive role of catheptic enzymes in the physiological processes of the intact organism has not yet been established, there is reason to believe that cathepsins have an important function in metabolism(7). It has been reported that alterations in catheptic activity, at least reflect, or correspond to variations in *in vivo* metabolism, even if the altered cathepsin ac-

tivity cannot be unreservedly interpreted as being the mechanism responsible for the changes observed *in vivo*(8-10). It is therefore of interest that during vit. E-deficiency there is an increase in proteolytic activity as measured by the method of Anson(5). Moreover, this increase appears to be confined to muscle, the only tissue in which obvious atrophy has occurred. These findings, at first sight, would appear to be at variance with those previously reported from this laboratory (1). However, the contrary results obtained by these 2 different methods might support the suggestion of Simpson(11) that there are at least 2 different mechanisms of protein catabolism. The data(1) obtained by the method of Kline(2) on the release of free amino nitrogen suggested a diminished rate of protein breakdown secondary to a decreased rate of synthesis; on the other hand, the increased proteolytic activity would suggest an accelerated rate of protein degradation. The observed dimunition of muscle mass *in vivo* may be produced by the resultant of the changes in rates of two different mechanisms involved in protein metabolism.

Summary. 1. The proteolytic activity of homogenates and extracts of muscles of rabbits with muscular dystrophy induced by vit. E-deficiency was assayed with denatured hemoglobin as substrate. 2. Vit. E-deficiency resulted in increased proteolytic activity per g wet weight of muscle and per mg of muscle protein nitrogen of both the extracts and homogenates. 3. No change in proteolytic activity of spleen, kidney or liver was observed during vit. E-deficiency.

1. Milman, A. E., Silides, D. N., and Milhorat, A. T., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 637.
2. Kline, D. L., *Endocrinology,* 1949, v45, 596.
3. Snoke, J. E., and Neurath, H., *J. Biol. Chem.,* 1950, v187, 127.
4. Goettsch, M., and Pappenheimer, A. M., *J. Exp. Med.,* 1931, v54, 145.
5. Anson, M. L., *J. Gen. Physiol.,* 1938, v22, 79.
6. Youden, W. J., *Statistical Methods for Chemists,* John Wiley & Sons, New York, 1951, p40.
7. Fruton, J. S., in Green, D. E., *Currents in biochemical research,* Interscience Publishers, Inc. New York, 1946, p123.
8. Maver, M. E., Dunn, T. B., and Greco, A., *J. Natl. Cancer Inst.,* 1945-46, v6, 49.
9. Mathies, J. C., Palm, L., and Gaebler, O. H., *Endocrinology,* 1951, v49, 571.
10. Maver, M. E., Greco, A. E., Løvtrup, E., and Dalton, A. J., *J. Natl. Cancer Inst.,* 1952, v13, 687.
11. Simpson, M. V., *J. Biol. Chem.,* 1953, v201, 143.

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Persistence of Group A Streptococci in Tissues of Rabbits after Infection.*† (21557)

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Group A streptococcal infections of the throat in man are symptomatically of short duration, but the host in a high percentage of cases continues to harbor the infecting organism in the tonsils or pharynx for a period of weeks or months(1). In view of the capacity of this organism to persist for long periods in the tissues of man, it seemed of interest to determine its capacity to persist in

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various tissues of an experimental animal.

Materials and methods. Rabbits. Albino hybrid rabbits of either sex weighing 1.5-2.5 kilos were employed in all experiments. *Streptococci.* Strains of type 1 or 24 group A streptococci were used in these studies. The strain to be used was grown for 18 hours in bacto-brain heart infusion (Difco) and various dilutions in this broth were used for experimental infections. All injections were made under sterile conditions into the marginal ear vein of the rabbit. *Cortisone.* A saline suspension of cortisone acetate (Merck) in a concentration of 25 mg per ml was injected intramuscularly in the thigh according to the schedules described below. *Blood cultures.* Blood was collected from the marginal ear vein or directly from the heart in sterile tubes containing 3% sodium citrate. Pour plates were made by mixing 1 ml of blood with bacto-tryptose blood agar base (Difco). Broth cultures were also made by adding small amounts of blood to 10 cc amounts of bacto-brain heart infusion. Pour plates were examined for 3 days and broth cultures for 10 days before discarding. *Organ cultures.* All animals were prepared by clipping and shaving the chest and abdomen and then sacrificed by a blow to the head. The skin was prepared by scrubbing with green soap and then applying tincture of iodine and 70% alcohol. Using aseptic technic the heart, one kidney, the spleen and part of the liver were removed. The organ to be cultured was cut into small pieces with scissors and the parts placed into 50 cc of bacto-brain heart infusion. These cultures were examined over a period of at least 10 days. *Grouping and typing of streptococci.* All strains of streptococci isolated were grouped and typed according to the method of Swift, Wilson and Lancefield(2).

Results. Persistence of group A streptococci in tissues of rabbits after infection. To determine if group A streptococci persisted in the heart, liver, or kidney after disappearance from the blood stream the following experiment was performed. Twenty-nine rabbits were given an intravenous injection of 1 cc of a broth culture of type I group A streptococcus. Nine of these animals died before the experiment could be completed, seven 48 hours after injection of the streptococci, one at 7 days and one at 10 days. Repeated blood cultures were obtained at intervals of 24 to 48 hours on the remaining animals. When at least 2 successive sterile blood cultures had been obtained, the rabbits were sacrificed in groups of 4, and the heart, liver and kidney cultured. Animals were sacrificed as early as 7 days and as late as 16 days after infection. Twenty-five per cent of the rabbits showed streptococci in one or more of the organ cultures. Two of these had had sterile blood cultures for 7 days before being sacrificed, 2 for 5 days, and one for 4 days. Three of the rabbits had had 4 sterile blood cultures, one 3, and one 2. The streptococci were isolated from one organ in 4 animals and in all 3 organs cultured in another. The heart was the site of localization in 2 instances, the kidney in 2 and the liver in 3. All strains of streptococci isolated were found to be group A, type 1, the same type as that used in infecting the rabbits.

Another experiment was now performed to determine if streptococci could persist for much longer periods after infection. Nineteen rabbits were sacrificed 35 days after the intravenous injection of 1 cc of a 10^{-1} dilution of an 18 hour broth culture of a group A, type 1 streptococcus. In this experiment cultures were made of the liver, heart, kidney and spleen. In addition, if there was swelling of any of the joints, the joints were opened under sterile conditions and material taken from the joint cultured. Blood cultures were taken immediately before sacrifice and were obtained by direct cardiac puncture. At least 5 cc of blood was cultured in 50 cc of brain heart infusion in each case. The results of this experiment are shown in Table I. Six of the 19 rabbits showed group A type 1 streptococci in one or more of the cultures taken. Two of the rabbits had streptococci in the blood and in 2 or more of the organs. Four of the rabbits showed streptococci in the material from the joints, 3 of these in conjunction with positive cultures elsewhere, and in one the joint was the only site of isolation. In one rabbit the liver and heart were the only sites of localization. In addition to the cul-

TABLE I. Isolation of Group A, Type I Streptococci from Rabbits 35 Days after Infection.

No. rabbits	Localization of streptococci*					
	Blood	Liver	Heart	Kidney	Spleen	Joint
13	0	0	0	0	0	0
1	+	+	+	0	+?†	+
1	0	+	+?†	0	0	—
1	+	+	+	+	0	—
1	0	+	0	0	0	+
1	0	+	C‡	0	+	+
1	0	0	0	0	0	+
19	2	5	3	1	2	4

* Initial infection established by intravenous injection of 1 cc of 10^{-1} dilution of 18 hr broth culture.

† These strains of beta hemolytic streptococci were lost before the group and type could be identified.

‡ Cultures from heart of this animal were contaminated.

tures of the blood and joints, the liver was the site of localization in 5 instances, the heart in 3 instances, the spleen in 2, and the kidney in one instance.

Reappearance of group A Streptococci in blood streams of rabbits treated with cortisone. It has been shown that cortisone treatment of the host enhances many acute infections in man and animals. Specifically, it has been demonstrated that cortisone greatly increases the pathogenicity of group A streptococci for the rabbit(3). After it had been shown that group A streptococci could persist in the organs of rabbits without dissemination into the blood stream, an experiment was designed to determine the effect of cortisone on the reestablishment of bacteremia in these animals.

Four rabbits were given an intravenous injection of 1 cc of a 10^{-1} dilution of an 18 hour broth culture of a group A type 24 streptococcus. Forty-seven days later, following 2 sterile blood cultures in each animal, intramuscular cortisone in the dosage of 25 mg per day was begun. Two days later blood cultures from 2 of the rabbits grew out beta hemolytic streptococci. The strain of streptococcus from one of the rabbits was lost before it could be classified. The strain isolated from the other rabbit proved to be a group A type 24 streptococcus. In this animal, 2 subsequent blood cultures on the 3rd and 7th days of cortisone treatment also contained type 24 streptococci. Cortisone was discontinued after 7 days. In the same 4 rabbits cortisone treatment in the dosage of 75 mg/day was resumed on the 98th day following infection and was continued for 9 days. On the 6th day of cortisone treatment the blood culture of one rabbit contained large numbers of group A type 24 streptococci. All cultures with one exception taken during the following 8 days contained the same strain of streptococcus. The blood cultures of the other 3 rabbits, including the one that had had a positive blood culture earlier, remained sterile. This experiment indicates that streptococci can persist in the tissues of rabbits for periods up to 104 days after infection. The part played by cortisone in the reestablishment of bacteremia is not clear, but the sterile blood cultures prior to treatment and the appearance of streptococci in the blood in large numbers after treatment suggest that cortisone was the responsible agent.

Discussion. Data presented emphasize the capacity of the group A streptococcus to remain viable in tissues for long periods, without blood stream dissemination. Persistence of streptococci was not correlated with demonstrable local tissue damage except for the joints, in which purulent arthritis was frequently encountered. Heart, liver and kidney tissues from which streptococci were cultured showed no evidence of suppurative infection.

It is of interest that Green(4) reported the isolation of group A streptococci from the heart valves of 8 of 9 patients dying with rheumatic endocarditis. In all patients the blood was sterile and in 5 of the 8 patients the same serological type of streptococcus as that isolated from the heart had been isolated from the pharynx before death. Collis(5) in the same year reported the isolation of hemolytic streptococci from 22 of 42 heart valves from 17 patients dying with rheumatic endocarditis. These strains of streptococci were not classified serologically and this report, therefore, does not carry the significance of Green's but is of great interest in itself. As in Green's study, cultures of the blood contained no streptococci. There were no signs of suppuration in any of the cases, the sole

lesion being that of rheumatic endocarditis. Thomson and Innes(6) in a continuation of Green's work, isolated hemolytic streptococci from the heart valves of 5 patients dying of acute rheumatic fever. Cultures of 11 of 26 valves, all showing acute rheumatic involvement, contained this organism. Cultures of heart blood or other organs in 3 of the cases also contained the same type of streptococcus as was isolated from the heart valves. The significance of these reports in regard to the pathogenesis of rheumatic fever has been a debatable point, but they suggest that in the human, as in the rabbit, streptococci may persist in tissues for long periods of time without bacteremia. Morris and his group from the Streptococcal Diseases Laboratory(7) have recently shown that penicillin therapy of streptococcal pharyngitis could be delayed until 9 days after the onset of the acute illness and still result in a significant reduction in the subsequent occurrence of rheumatic fever. The antibody response of these patients was only slightly inhibited. These data would seem to indicate that the development of rheumatic fever depends upon the persistence of the group A streptococcus and not necessarily on the antigen-antibody reaction.

The reappearance of streptococci in large numbers in the circulating blood following cortisone administration is of some interest. The observation suggests that cortisone has, in addition to its well-documented capacity to enhance acute bacterial infection, the property of reestablishing the systemic dissemination of latent streptococcal infection.

Summary. Group A streptococci persisted in the tissues of rabbits, in the absence of bacteremia, for periods up to 104 days following infection. Except in the case of suppurative arthritis such persistence was not associated with any demonstrable pathological lesion. Cortisone might have played a part in reestablishing a bacteremia in rabbits long periods after the acute infection. The significance of these findings in relation to the pathogenesis of rheumatic fever is discussed.

1. Streptococcal Diseases Laboratory, unpublished data.

2. Swift, H. F., Wilson, A. T., and Lancefield, R. C., *J. Exp. Med.*, 1943, v78, 127.

3. Mogabgab, W. J., and Thomas L., *J. Lab. and Clin. Med.*, 1952, v39, 271.

4. Green, C. A., *Ann. Rheumatic Dis.*, 1939, v1, 86.

5. Collis, W. R. F., *Lancet*, 1939, v237, 817.

6. Thomson, S., and Innes, J., *Brit. Med. J.*, 1940, v2, 733.

7. Morris, A. J., Catanzaro, F. J., Chamovitz, R., Stetson, C. A., and Rammelkamp, C. H., *Clin. Research Proc.*, 1954, v2, 75.

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Effect of Aminopterin and Certain Purine Antagonists on Adenine Nucleotide Levels in Tissues of Tumor-Bearing Mice.* (21558)

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In 3 previous papers(1,2,3), nucleotide levels in blood and other tissues of normal and tumor-bearing mice under various experimental conditions were recorded. Since certain purine as well as folic acid antagonists are known to affect, at least temporarily, some types of neoplastic growth, and since it is presumed that such effects are mediated somewhere in the chain of nucleotide metabolism, experiments were designed to measure quantitatively the influence of several purine antagonists and also of a folic acid antagonist on adenine nucleotide levels in tissues of tumor-bearing mice.

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Materials and methods. Purine antagonists

TABLE I. AA, ADP, ATP, and Total Nucleotide Content of Liver Tissue Taken from Mice Bearing 6-Day-Old Sarcoma 180. Mean values expressed as mg % adenine, with standard errors. For statistical test, dosage, and injection data, see text.

Treatment	N	AA	ADP	ATP	Total nucleotide	Sum (AA, ADP, ATP)
Control	50	10.9 ± .53	4.0 ± .60	12.2 ± .68	57.7 ± 1.21	26.7 ± .83
6-mercaptopurine	10	10.2 ± 1.00	2.4 ± .72	8.5 ± 1.06*	48.3 ± 1.50*	21.1 ± 1.24*
8-azaguanine	9	8.2 ± 1.84	5.1 ± 2.05	15.0 ± 2.07	62.5 ± 1.58*	28.2 ± 2.38
2,6-diaminopurine	7	10.2 ± 1.02	4.0 ± 1.31	11.1 ± 2.03	53.8 ± 2.64	25.3 ± 1.74
Aminopterin	15	10.1 ± .75	3.4 ± .79	14.5 ± .88*	57.4 ± 1.40	28.1 ± 1.22
Aminopterin + adenylic acid	11	12.9 ± .98	5.4 ± 1.07	9.3 ± 1.20*	56.6 ± 1.99	27.6 ± 1.34

* Difference between this and control values at top of column is statistically significant.
N = No. of determinations made.

selected were 2, 6-diaminopurine, 6-mercaptopurine, and 8-azaguanine†; the folic acid antagonist was 4-aminopteroyl-glutamic acid (Aminopterin, Lederle Lot. No., 3001C-10).‡ In one experiment, aminopterin and adenylic acid were injected simultaneously; in another, folic acid was tested for its independent effect on nucleotide levels. These compounds, in fine-ground aqueous suspension and in amounts approximating dosages described in the literature on mouse chemotherapy experimentation, were injected into mice bearing 6-day-old implants of sarcoma 180. Female Swiss mice of standard age and weight were used throughout. Each experiment involved the use, except where otherwise indicated, of two .5-ml intraperitoneal injections, one made 18 hrs and the other 2 hrs before sacrifice of animal for tissue assay. Dry weight content of each injection for each compound was: 6-mercaptopurine—.2 mg; 8-azaguanine—2.8 mg; 2, 6-diaminopurine—1.6 mg; aminopterin—.5 mg; adenylic acid—5 mg (intravenous in .2 ml water); folic acid—1 mg. Animals were killed by cervical fracture; then as quickly as possible tissues were excised and dropped into liquid nitrogen. The central necrotic core of each tumor used was removed and discarded. Tissues were often taken from multiple donors of the same experimental class to yield a wet weight aliquot of about 1 g. For muscle assay, a rectangular section from ventral abdominal musculature was used. The frozen tissues were pulverized and extracted once with a 5-ml aliquot of cold 10% trichloracetic acid and twice with 2-ml aliquots of 5% trichloracetic acid. After centrifugation, extracts were combined, neutralized, made to 10 ml volume, treated with .5 ml of 25% barium acetate and 4 volumes of ethanol, and placed in cold room overnight. Barium precipitates were collected by centrifugation, dissolved in dilute HCl, freed of barium with Na_2SO_4, neutralized, made to volume and assayed enzymatically for adenine attributable to adenylic acid (AA), adenosine diphosphate (ADP), and adenosine triphosphate (ATP), by methods described earlier(1). Total nucleotide was determined spectrophotometrically from the 2600 Å reading.

The *results* are summarized in Tables I-V, where number of determinations made are shown together with mean levels of nucleotides measured; also the standard error of the mean. If the difference between means exceeded the S. $E_{diff.}$ by 2 or more times (see formula, p. 232(3)), the difference is considered significant and so indicated with an asterisk. In all cases, comparison is made with values derived from uninjected tumor-bearing control animals.

In *liver* tissue, Table I, 6-mercaptopurine lowers the level of ATP significantly, as well as total nucleotide and the sum of known nucleotides. Lowering of the sum of known nucleotides (last column) is largely a reflection of the lowering of ATP. 8-Azaguanine raises total nucleotide by a small but significant amount, but this rise is not due to an increase in any of the nucleotides measured

† Supplied through kindness of Dr. G. H. Hitchings, Wellcome Research Laboratories, Tuckahoe, N. Y.

‡ Supplied through kindness of Dr. H. P. Broquist, Lederle Research Laboratories, Pearl River, N. Y.

TABLE II. AA, ADP, ATP, and Total Nucleotide Content of Spleen Tissue Taken from Mice Bearing 6-Day-Old Sarcoma 180.

Treatment	N	AA	ADP	ATP	Total nucleotide	Sum (AA, ADP, ATP)
Control	52	7.4 ± .46	2.8 ± .40	16.5 ± .56	51.3 ± .73	26.7 ± .69
6-mercaptopurine	9	6.5 ± 1.06	2.4 ± .47	15.2 ± .67	51.3 ± 1.45	24.2 ± 1.01*
8-azaguanine	9	7.5 ± 1.23	.5 ± .31*	20.7 ± 1.23*	55.2 ± 2.62	28.6 ± 1.85
2,6-diaminopurine	7	4.6 ± .61*	3.2 ± .78	17.1 ± .97	50.8 ± 1.67	24.9 ± 1.17
Aminopterin	14	5.8 ± .80	1.2 ± .64*	18.7 ± 1.14	49.8 ± 1.25	25.8 ± 1.45
Aminopterin + adenylic acid	11	7.4 ± .75	3.1 ± .79	15.8 ± 1.47	48.2 ± 1.34	26.0 ± 1.66

* See footnote, Table I.

and must therefore represent nucleotide of presently unknown identity. 2, 6-Diaminopurine has no effect on the level of liver nucleotides. Aminopterin raises the ATP level, while adenylic acid, which by itself was found earlier(3) to raise the ATP level in liver, lowers it when given simultaneously with aminopterin.

In *spleen* tissue (Table II) 6-mercaptopurine effects on separate known nucleotide levels are not statistically significant, while the sum of the 3 changes is. 8-Azaguanine changes the ADP and ATP but not the total nucleotide levels; 2, 6-diaminopurine produces no effect in spleen other than a lowering of the AA. Aminopterin depresses the ADP level; when given with adenylic acid it appears, as in liver, to prevent the rise of spleen ATP expected from adenylic acid administered alone (3).

In *muscle* tissue (Table III), 6-mercaptopurine induces a lowering of ADP and ATP as well as of total nucleotide and the sum of known nucleotides. 8-Azaguanine does not affect muscle nucleotide levels, nor do 2, 6-diaminopurine or aminopterin. Aminopterin with adenylic acid, however, significantly changes the levels of AA, ATP, total nucleotide, and the sum of known nucleotides.

In *tumor* tissue (Table IV), 6-mercaptopurine lowers the sum of known nucleotides without, however, reflecting itself in individual values. 8-Azaguanine raises total nucleotide, while 2, 6-diaminopurine has no effect. After aminopterin administration, the most striking effect seen during these experiments is displayed: a lowering by about 50% of the ATP in tumor tissue. Total nucleotide as well as the sum of known nucleotides are also considerably lowered. When adenylic acid is administered concurrently with aminopterin, the depression of ATP level, as well as total nucleotide and the sum of known nucleotides, is more pronounced than when either adenylic acid(3) or aminopterin is used alone.

The effect of aminopterin on tumor nucleotide raised the question as to whether this might be due to folic acid which frequently contaminates preparations of aminopterin. To appraise this possibility, a separate experiment was performed using folic acid. The outcome, summarized in Table V, indicates that injected folic acid has litle effect on nucleotide levels of tissues examined. Parenthetically, control values in Table V vary

TABLE III. AA, ADP, ATP, and Total Nucleotide Content of Muscle Tissue Taken from Mice Bearing 6-Day-Old Sarcoma 180.

Treatment	N	AA	ADP	ATP	Total nucleotide	Sum (AA, ADP, ATP)
Control	49	2.4 ± .24	2.3 ± .39	54.2 ± 1.22	71.3 ± .78	58.9 ± 1.29
6-mercaptopurine	10	2.3 ± .38	1.3 ± .27*	43.9 ± 1.88*	63.4 ± 1.37*	48.5 ± 1.42*
8-azaguanine	9	2.2 ± .52	1.4 ± .37	54.0 ± 3.36	70.9 ± .96	57.5 ± 3.52
2,6-diaminopurine	6	1.6 ± .58	3.0 ± 1.3	50.0 ± 3.58	67.1 ± 2.26	54.5 ± 3.06
Aminopterin	14	2.8 ± .33	1.1 ± .52	55.4 ± 1.91	72.9 ± 1.50	59.4 ± 1.69
Aminopterin + adenylic acid	9	3.2 ± .31*	2.3 ± 1.12	43.4 ± 2.28*	59.8 ± 1.54*	48.9 ± 1.29*

* See footnote, Table I.

TABLE IV. AA, ADP, ATP, and Total Nucleotide Content of Tumor Tissue Taken from Mice Bearing 6-Day-Old Sarcoma 180.

Treatment	N	AA	ADP	ATP	Total nucleotide	Sum (AA, ADP, ATP)
Control	51	3.3 ± .23	1.9 ± .31	10.2 ± .35	39.9 ± .93	15.4 ± .48
6-mercaptopurine	9	3.1 ± .33	1.2 ± .31	8.8 ± .78	37.8 ± 2.23	13.2 ± .74*
8-azaguanine	9	3.2 ± .45	1.7 ± 1.05	11.9 ± 1.18	47.5 ± 1.79*	16.7 ± 1.43
2,6-diaminopurine	7	3.1 ± .70	2.0 ± .70	10.3 ± .69	40.8 ± 2.20	15.4 ± .83
Aminopterin	16	1.7 ± .36*	1.2 ± .33	5.5 ± .61*	27.3 ± 1.78*	8.4 ± .65*
Aminopterin + adenylic acid	11	3.0 ± .26	1.5 ± .58	2.8 ± .52*	20.6 ± .96*	7.4 ± .63*

* See footnote, Table I.

somewhat from those listed in the other Tables. We have no explanation for this, other than that the experimental work upon which Table V is based was done at a different time of year than that upon which Tables I-IV is based. The possibility of seasonal or other periodic variations in tissue nucleotide levels emphasizes the need to employ controls for each experimental manipulation. Our adherence to this principle is reflected in the relatively large number of control determinations summated in Tables I-IV.

The effect of combined aminopterin and adenylic acid is interesting in relation to the concept of "concurrent blocking" proposed by Elion, Singer, and Hitchings(4), and also to the earlier concept of "sequential blocking" of Potter(5). Additionally, the work of Skipper and his co-workers is relevant in this connection(6).

Further consideration of biochemical mechanisms involved in the effects reported here will not be undertaken at present, other than to note the possibility that such metabolic antagonists, singly or in combination, may affect not only the chain of purine synthesis but also the purine moiety of adenosine co-enzymes.

The clue provided by the present data—*i.e.*, the effect of concurrently administered aminopterin and adenylic acid on ATP and total nucleotide in tumor tissue—is serving as the basis for continued studies. The other changes asterisked in the Tables as statistically significant are, in many cases, of insufficient magnitude to justify conclusions of firm metabolic significance.

Summary. 1. Tumor-bearing mice were subjected to short-term treatment with various purine and folic acid antagonists, then sacrificed for quantitative assay of tissue adenine nucleotides. 6-Mercaptopurine somewhat lowered the levels of ATP, total nucleotide, and the sum of known nucleotides in liver and muscle, and to a lesser extent in spleen and tumor tissues. 8-Azaguanine raised total nucleotide levels in liver and tumor tissue, but not in muscle or spleen. 2, 6-Diaminopurine was essentially without ef-

TABLE V. AA, ADP, ATP, and Total Nucleotide Content of Tissues Taken from Control and Folic Acid-Treated Mice Bearing 6-Day-Old Sarcoma 180.

Tissue	N	AA	ADP	ATP	Total nucleotide	Sum (AA, ADP, ATP)
(Control)						
Liver	8	6.6 ± .68	2.0 ± .87	8.6 ± .92	40.2 ± 1.36	17.2 ± .90
Spleen	8	3.2 ± .76	2.1 ± .90	11.4 ± 1.11	38.0 ± 1.60	16.7 ± 1.59
Muscle	7	2.1 ± .23	.6 ± .39	48.2 ± 2.45	60.9 ± 1.82	50.9 ± 2.22
Tumor	10	2.1 ± .38	1.2 ± .52	6.6 ± .44	29.6 ± .94	9.9 ± .74
(Folic acid-treated)						
Liver	9	5.6 ± .67	2.3 ± .96	9.5 ± 1.07	42.9 ± 1.99	17.4 ± 1.06
Spleen	10	3.9 ± .51	2.0 ± .47	11.8 ± .53	40.8 ± 1.71	17.7 ± .64
Muscle	10	3.8 ± .59*	.9 ± .36	48.6 ± 1.80	64.5 ± 1.30	53.3 ± 1.84
Tumor	8	2.4 ± .51	.8 ± .44	7.0 ± .36	33.4 ± 1.47*	10.2 ± .71

* See footnote, Table I.

fect on nucleotide levels in these tissues. 2. Aminopterin elevated slightly the ATP level in liver but was without effect on ATP in spleen or muscle. In tumor tissue, aminopterin reduced the ATP level by nearly 50% and, when used concurrently with adenylic acid, by nearly 70%.

1. Zahl, Paul A., and Albaum, Harry G., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 388.

2. Albaum, Harry G., and Zahl, Paul A., *ibid.*, 1953, v82, 337.

3. ———, *ibid.*, 1954, v85, 231.

4. Elion, G. B., Singer, S., and Hitchings, G. H., *J. Biol. Chem.*, 1954, v208, 477.

5. Potter, V. R., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 41.

6. Skipper, H. E., Thomson, J. R., and Bell, M., *Cancer Res.*, 1954, v14, 503.

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Changes in Lipid and Electrolyte Concentrations in Adrenalectomized Rats After Alloxan. (21559)

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It has been adequately shown that in alloxan diabetic animal the beta-islet cells are damaged and there is reduction of the insulin content in the pancreas(1). However, certain metabolic function tests performed on alloxanized animals indicate that lack of insulin alone can not account for the wide divergencies observed in the fasting blood sugar level, the types of glucose tolerance curves, and lack of relationship between symptoms when fasted and extent of hyperglycemia when not fasted(2). The changes in blood glucose levels after alloxan are well documented(3,4) but little is known concerning the effect of this drug upon other tissue chemical constituents nor is it known what factors may be responsible. Pertinent to this problem was the demonstration by Goldner and Jauregui(5) that alloxan inhibited spontaneous glycolysis in the liver as well as adrenalin-induced glycolysis. Recently, Dury(6) showed that soon after alloxan administration in intact rats the concentrations of liver lipid components, and plasma and liver electrolytes were significantly altered. Collateral data in that study indicated the alterations in tissue constituents during the first 24 hours after alloxan were in the nature of "stress" types of responses and might be adrenocortically conditioned. This paper reports the results of experiments on adrenalectomized rats.

Materials and methods. Male Wistar rats weighing 275-325 g were adrenalectomized 3-4 days before intravenous alloxan was administered. The rats were given a 1% NaCl solution for drinking purposes and laboratory chow *ad libitum*. A 5% solution of alloxan (Eastman #1772) was injected at the value of 40 mg/kg body weight directly into an exposed femoral vein in all experimental animals. Control rats received an equal quantity of physiological saline (0.1 ml/100 g body weight) and sacrificed for tissue specimens 4 hours later. The results are reported on groups of rats comprising 2 series as follows: Series I—Rats sacrificed at 4 and 74 hours after alloxan; Series II A—Groups of rats maintained with 1% NaCl for drinking purposes and in addition given daily injections of 0.1 ml Lipo-adrenal extract (Upjohn) from the day of adrenalectomy to sacrifice at 4, 24, and 72 hours after alloxan administration. Series II B—Groups of rats similarly maintained and treated as above but given a subcutaneous injection of 0.2 ml Lipo-adrenal 30 minutes before alloxan administration. These animals were sacrificed at 4 and 24 hours after alloxan (the latter also received an additional 0.2 ml Lipo-adrenal 4 hours before being sacrificed). The rats sacrificed 72 hours after alloxan were fasted approximately 20 hours before the drug was administered but food and salt solu-

tion were available *ad libitum* from then until they were sacrificed. The animals in the other groups were not fasted before alloxan injection. Ether anesthesia was used for all surgical procedures, intravenous injections and when the tissues were taken for chemical analyses. Heart blood was obtained from the anesthetized rat by cardiac puncture in a syringe moistened with heparin. The blood was immediately centrifuged and the plasma removed for chemical determinations. Sections of liver and gastrocnemius muscle were obtained for glycogen(7,8) and electrolytes determinations. The sodium and potassium content of liver, plasma and muscle were determined with the aid of an internal lithium standard flame photometer.* The electrolytes of muscle and liver were determined on samples of dried, defatted tissue after ashing at 425°C overnight. The values are represented as meq. per 100 g fat-free solids. The details of the methods employed for liver lipids analyses at this laboratory were described previously(9). Glucose in plasma was determined by the photometric method of Nelson(10).

Results. Tissue constituents in adrenalectomized rats maintained with drinking solution of 1% NaCl. The concentrations of various tissue constituents in groups of rats sacrificed at 4 and 72 hours after alloxan and in controls are presented in Table I. These data show that alloxan had not induced any alterations in the concentrations of the liver lipid fractions of the group 4 hours after administration of the drug, nor any of the other constituents except a significantly decreased concentration of liver potassium. In the 72 hour post-alloxan group, the concentrations of liver total cholesterol and its fractions, liver and muscle potassium, and plasma glucose were significantly elevated; whereas liver and muscle glycogen concentrations were significantly lower than control values.

2. *Tissue constituents in adrenalectomized rats maintained with 1% NaCl and daily small doses of Lipo-adrenal.* The concentrations of various tissue constituents in groups of rats at 4, 24, and 72 hours after alloxan and controls are presented in Table II, Part A. The concentrations of liver lipid fractions in rats 24 hours post-alloxan were not different from respective control mean values. In the 4- and 72-hour post-alloxan groups, the concentrations of liver total cholesterol and its fractions were significantly different from the controls but the concentrations of total lipids, phospholipids and neutral fat did not deviate from control values. The blood glucose levels in these two groups were significantly elevated, and the liver glycogen content was significantly decreased in the 72-hour post-alloxan group.

The data in Part B, Table II show the alterations in concentrations of various chemi-

TABLE I. Concentrations of Chemical Constituents in Tissues of Adrenalectomized Rats Maintained with 1% NaCl Solution.

Tissue constituents	Controls 8	After alloxan*
Liver lipid fractions (g %)		
Total lipids	4.71 ± .08†	5.17 (4.16–5.83)‡
PLP	4.32 ± .12	4.74 (4.25–5.31)
Total chol.	.24 ± .006	.32 ± .02 ** b
Free "	.22 ± .005	.24 ± .003 †† b
Ester "	.05 ± .005	.08 ± .02 ** b
Neutral fat	.17 ± .05	.31 (.10–.96)
Liver glycogen, g %	1.74 ± .07	.028 ± .01 ** b
Muscle glycogen	.25 ± .01	.200 ± .02 †† b
K:		
Plasma, meq/L	5.57 ± .10	5.95 (4.74–8.52)
Liver, " §	28.4 ± .6	25.3 ± .6 ** a 31.8 ± 1.1 †† b
Muscle, "	40.8 ± .7	44.4 ± .9 ** b
Na:		
Plasma, meq/L	139.4 ± .7	140.2 (137–142.2)
Liver, " §	7.7 ± .4	7.6 (5.8–10.3)
Muscle, "	9.1 ± .7	9.8 (7.1–11.7)
Plasma glucose, mg %	177. ±6.	366. ± 95. ** b

* Groups of rats 4 hr (a), and 72 hr (b) after alloxan, 8 and 5 animals in each group, respectively.

† Mean ± S.E.

‡ Where the concentrations of the various constituents in each exp. group were not different from the mean value in control group then the combined mean value and range in groups (a) and (b) is given; where an individual value in a group was different from controls then the mean ± S.E. is given. "P" value .05-.01 indicated by ††; and 0.01 and less indicated by **.

§ Values expressed/100 g fat-free solids.

* Flame photometer used is of the type described by Berry, Chappell, and Barnes (*Indust. & Eng. Chem.*, Analyt. Ed., 1946, v18, 19). The particular model was made by Mr. Chappell.

TABLE II. Concentrations of Chemical Constituents in Adrenalectomized Rats Receiving Lipo-Adrenal Extract.

Tissue constituents	Controls 8	Series II A after alloxan*	Series II B after alloxan†
Liver lipid fractions (g %)			
Total lipids	5.38 ± .08‡	5.32 (3.82–6.81)‡	5.37 (4.58–5.49)
PLP	4.55 ± .12	4.65 (3.43–6.14)	3.92 (3.56–4.28) ** a,b
Total chol.	.25 ± .005	.23 ± .005 †† a .32 ± .01 ** c	.24 (.20– .27)
Free "	.18 ± .007	.20 ± .004 †† a .24 ± .006 ** c	.21 (.18– .27) †† a,b
Ester "	.07 ± .008	.03 ± .004 ** a	.03 (.01– .06) ** a,b
Neutral fat	.37 ± .37	.37 (.04–.89)	1.09 (.70–1.57) ** a,b
Liver glycogen§	1.73 ± .23	.075 ± .03 ** c	.41 ± .22 ** b
Muscle "	.23 ± .008	.25 (.15–.33)	——
K:			
Plasma meq	5.06 ± .14	5.56 (4.44–8.36)	6.92 ± .34 ** b
Liver "	30.6 ±1.2	30.5 (23.5–41.1)	35.2 ± .8 ** b
Muscle "	40.0 ±1.0	43.4 ± .6 †† b	43.0 ± .4 †† b
Na:			
Plasma "	141.5 ± .07	141.5 (135.0–147.2)	139.6 (136.2–145.2)
Liver "	8.6 ± .4	6.7 ± .3 ** a	8.8 (7.2–11.0)
Muscle "	9.3 ± .5	9.2 (7.3–13.8)	9.1 (6.7–10.1)
Plasma glucose	177. ±6.	262. ± 31. ** a 415. ± 39. ** c	250. ± 28 †† a

* Groups of rats 4 hr (a), 24 hr (b), and 72 hr (c) after alloxan, 8 rats in each group.
† Groups of rats 4 hr (a), and 24 hr (b) after alloxan, 6 rats in each group. See text for lipo-adrenal dosage and timing.
‡ See legend, Table I.
§ Concentrations of various constituents expressed same as in Table I.

cal constituents in groups of rats after alloxan which were maintained as those above but had received 0.2 ml Lipo-adrenal (twice the regular level) approximately 4 hours before being sacrificed. The concentrations of liver lipid components in both groups (4 hours and 24 hours post-alloxan) presented a similar picture: Phospholipid concentrations significantly decreased, free cholesterol elevated, and ester cholesterol concentrations significantly depressed. The neutral fat concentration in both alloxan-treated groups was significantly increased (approximately 200%). The blood glucose level in the 4-hour group was significantly elevated but not different from the control value in the 24-hour group. The concentration of liver glycogen 24 hours post-alloxan was markedly depressed, whereas the concentration of potassium in plasma, liver, and muscle was significantly elevated.

Discussion. In a previous report(6) it was shown that at 4 and 24 hours following alloxan administration in intact rats marked changes occurred in the concentration of certain chemical constituents of plasma and liver, particularly in the liver lipid fractions. It is evident from the data presented here (Tables I and IIA) that this was not the case in the adrenalectomized rat. Furthermore, the administration of small doses of Lipo-adrenal extract had not influenced this lack of "responsiveness" to alloxan. This is noteworthy, especially in the group 72 hours after alloxan, since the blood glucose in these animals was significantly greater than in controls. It is well-established that the occurrences of the primary and final phases of hyperglycemia observed after alloxan injection are dependent upon the function of the adrenal cortex since this condition is ameliorated by adrenalectomy(11,12) or chemical ablation of the zona reticularis and fasiculata of the adrenal cortex(4). The data presented here indicate that in intact rats the adrenal cortex was functionally involved in the alterations in concentration of liver lipid components, and the sodium and potassium content of plasma, liver, and muscle after alloxan ad-

ministration. This interpretation appears probable from the results found in the adrenalectomized groups given twice the maintaining level of Lipo-adrenal (Table II, Part B) in the attempt to simulate a condition of increased available adrenocortical hormone between the time of alloxan injection and sacrifice of the animals. The data indicate that this relatively small change in hormonal status of these rats was sufficient to influence the nature and extent of change in tissue chemical constituents. The results presented here and in the intact rat(6) strongly suggest that the adrenal cortex is involved in the mechanism of early changes in concentration of chemical components of tissues after alloxan, and may be involved in the pathologic-biochemistry of continued alloxan diabetes.

Summary. 1. Concentrations of liver lipid components, and liver, muscle and plasma electrolytes, glycogen and glucose were determined at 4, 24, and 72 hours after alloxan injection in adrenalectomized groups of rats. One series was maintained with 1% NaCl and a second series received daily injections of small levels of Lipo-adrenal extract in addition to the saline. In both series a moderate hyperglycemia was extant at 72 hours after alloxan; but at 4 and 24 hours the blood glucose levels were only slightly or not different from the controls. This was in marked contrast to the hyperglycemia found in intact rats at these intervals after alloxan. 2. In contrast to the changes in concentration in liver lipid components and liver and plasma electrolytes found in intact rats after alloxan at these periods, adrenalectomy apparently effectively prevented their occurrence. 3. An increase in the exogenously administered dose of adreno-cortical material just before alloxan injection resulted in significant alterations in liver lipid components as well as increased potassium concentration in plasma, muscle, and liver.

1. Wrenshall, G. A., Collins-Williams, J., and Best, C. H., *Am. J. Physiol.,* 1950, v160, 228.

2. Beach, E. F., Bradshaw, P. J., and Blatherwick, N. R., *Endocrinol.,* 1952, v50, 212.

3. Lukens, F. D. W., *Physiol. Rev.,* 1948, v28, 304.

4. Nichols, J., and Sheehan, H. L., *Endocrinol.,* 1952, v51, 362.

5. Goldner, M. G., and Jauregui, R. H., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 116.

6. Dury, A., *ibid.,* 1954, v86, 646.

7. Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.,* 1933, v100, 485.

8. Somogyi, M., *ibid.,* 1945, v160, 61.

9. Dury, A., and Treadwell, C. R., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 719.

10. Nelson, N., *J. Biol. Chem.,* 1944, v153, 375.

11. Long, C. N. H., and Lukens, F. D. W., *J. Exp. Med.,* 1936, v63, 465.

12. Kirschbaum, A., Wells, L. J., and Molander, D., PROC. SOC. EXP. BIOL. AND MED., 1945, v58, 294.

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Porphobilinogen and δ-Amino Levulinic Acid in Acute Porphyria. (21560)

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Acute porphyria is an inheritable metabolic disease characterized by attacks of abdominal pain, nervous symptoms, and the excretion of porphobilinogen(1). In the acute phase of the disease much porphobilinogen is excreted. However, during remission some individuals who have the disease may excrete little or no porphobilinogen(2). Others, during remission may continue to excrete 20-50% of the porphobilinogen which was excreted during relapse. Several lines of evidence suggest that the liver is the organ primarily involved (2).

The opportunity was presented to study the quantitative aspects of porphobilinogen excretion in a case of acute porphyria. At the same time, experiments were made to determine whether δ-amino levulinic acid, a precursor of porphobilinogen(3,4) might also be excreted in the urine in this disease. In addition to the

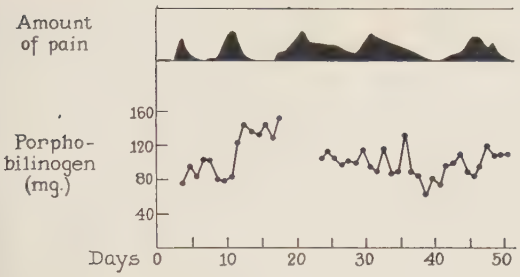

FIG. 1. mg porphobilinogen excreted daily in urine of a patient, with acute porphyria. The amount of pain is estimated as proportional to the amount of demerol required for sedation.

above data, a few other preliminary experiments are here recorded.

Experimental. The daily output of porphobilinogen in the urine of L.W., a 35-year-old female, is remarkably high, on the average 80-120 mg/day (Fig. 1). This rate of excretion remained high during periods in which only mild symptoms or no pain was observed. The onset of pain cannot be predicted by following the daily porphobilinogen excretion. The porphobilinogen in the urine was determined directly with Ehrlich's reagent (2% dimethyl amino benzaldehyde in 6.0 N HCl). Because of the relatively high concentration of porphobilinogen, the urine could be diluted sufficiently to avoid interfering reactions that would be caused by other urine constituents(5). In a 10 cc volumetric flask were added 0.1 cc urine, 4.9 cc H_2O, and 5.0 cc of Ehrlich's reagent. A color developed whose density between 0.05 and 0.10 in a cell 1 cm long was read at 555 mμ in a Beckmann spectrophotometer exactly 5 minutes after adding the reagent. The color which is developed from the porphobilinogen is equivalent to a molar extinction coefficient of 36,000 under these conditions (where the molar extinction $= \log 10 \frac{I_o}{I} \cdot \frac{1}{\text{cm x mole/l}}$).

The presence of δ-amino levulinic acid in urine of acute porphyria patients has not been reported previously. Preliminary studies indicated that in the urine of L.W., δ-amino levulinic acid may be excreted to the extent of about 40% by weight of the porphobilinogen excreted, *i.e.*, one day's output was approximately 40 mg. The method for δ-amino levulinic acid determination depends on the removal of porphobilinogen from the urine by passing the urine through a column of Dowex-2x2 resin as the acetate. The eluate then contains the δ-amino levulinate, urea and amino acids. The eluate is now passed through a column of Dowex-50x8 resin as the chloride. Urea is removed from the resin by elution with water. Then the δ-amino levulinic acid is eluted from the resin with 0.5 M phosphate buffer, pH 7.0. The eluate containing the δ-amino levulinic acid is coupled with acetyl acetone at pH 4.6 by heating the mixture in a boiling water bath for 10 minutes. A pyrrole compound is thus formed which gives a color with an equal volume of the Ehrlich reagent and is read at 555 mμ five minutes after mixing; with a density below 0.2 in a cell 1 cm long, the molar extinction is 24,500.

The high level of porphobilinogen excreted by L.W. during periods when no outward symptoms of pain were present is suggestive that porphobilinogen, *per se,* is not toxic. To test whether δ-amino levulinic acid might be toxic, 20 mg of this compound were injected intraperitoneally into a white rat of 150 g-weight. No symptoms of distress were observed. The next day the urine was found to contain a small amount of a pigment which appeared to be a zinc porphyrin. In ether the pigment had absorption bands at 575 and 535 mμ. When this ether solution was extracted with 3 N HCl the aqueous solution now contained 2 bands at 548 and 590 mμ typical for a porphyrin in acid solution. These properties suggest that the urinary pigment was a zinc porphyrin lacking vinyl groups(6,7). The contents of the stomach and duodenum and bone marrow of the femurs were found to fluoresce strongly pink in ultraviolet light which was suggestive of the presence of porphyrins. By the following procedure the porphyrin in the stomach and duodenum was identified as protoporphyrin: The stomach and duodenum together with their contents were ground in 15 cc glacial acetic acid, the suspension was filtered and the solution was extracted into ether. The ether phase was washed with water. The ether phase contained a pigment with a typical 4-banded ab-

sorption spectrum of a neutral porphyrin. The porphyrin was extracted with aqueous HCl. The HCl number(6) was determined to be 2.0, which indicated that the porphyrin was protoporphyrin. The absorption spectrum of the porphyrin in 1 N HCl was measured in a Cary spectrophotometer. Two absorption bands were found at 556 and 598 mμ, typical of acid protoporphyrin.

Since the liver may be primarily involved in the disease, acute porphyria(2), the liver should be capable of forming porphobilinogen from δ-amino levulinic acid. To test this idea qualitatively a suspension of guinea pig liver cells was used which was washed free of erythrocytes. Approximately 1 cc of this suspension was incubated in 5 cc Hank's salt solution for 17 hours in the presence of 4 mg δ-amino levulinate and 4 mg streptomycin, aerobically on a roller tube at 37°C. After 6 hours a pink fluorescence in ultraviolet light was observed in those tubes containing δ-amino levulinate and the liver cells, thus indicating the presence of porphyrins. After 17 hours sufficient porphyrins had been formed so that the typical absorption bands of the porphyrin mixture could be distinguished. The porphyrins were not further identified. A trichloracetic filtrate was prepared from one of these incubated tubes and was found to give a positive Ehrlich reaction thus indicating the presence of porphobilinogen. In the control tubes which lacked δ-amino levulinic acid or which contained δ-amino levulinic acid but which lacked the tissue, no red fluorescence, no absorption bands of porphyrins and no positive Ehrlich were observed. Thus it is concluded that a suspension of guinea pig liver cells has the ability to convert δ-amino levulinate through porphobilinogen to porphyrins.*

Discussion. Porphobilinogen has been shown to be a precursor of protoporphyrin and of heme(8,9). The large amounts of porphobilinogen and of δ-amino levulinic acid excreted by the acute porphyria patient L.W., may be ascribed to one of two causes. Either there is an excessive rate of formation of these compounds by the liver, or, the rate of formation is normal but there is a partial block above the porphobilinogen step so that porphobilinogen is not converted to porphyrin at a normal rate.

If it is assumed that acute porphyria is due to a partial block above the porphobilinogen step, then the rate of excretion of porphobilinogen in this patient may be a measure of the rate of production of protoporphyrin and of heme in normal liver. On such an assumption, the human liver would be capable of producing more than 100 mg of heme per day which would thence be converted to bile pigment. The heme incorporated into newly formed red cells is about 300 mg per day. Thus the liver would produce roughly one-fourth of the total heme.

London *et al.*(10) and Gray *et al.*(11) found that some 10-20% of the bile pigment, stercobilin, is derived from a source other than the old circulating erythrocytes. If the above hypothesis is correct, that some 100 mg of heme are produced daily by the liver and this heme is converted to bile pigment, it would explain the large amount of early labelled stercobilin which appears in the feces after feeding of labelled glycine.

Summary. Quantitative studies on urine of an acute porphyria patient reveal a daily output of about 100 mg porphobilinogen and about 40 mg of δ-amino levulinate. This high rate of production suggests the possibility that the normal liver may convert these compounds to heme and thus may produce approximately 100-140 mg of heme per day. This heme might be then, the source of the early labelled stercobilin observed by London and by Gray.

* While this paper was in press a note appeared by K. D. Gibson, A. Neuberger, and J. J. Scott (*Biochem. J.*, v58, xli, 1954) describing the isolation of an enzyme from liver which converts δ-amino levulinic acid to porphobilinogen.

1. Waldenström, J., *Acta med. Scandinav.*, 1937, supp. 82.

2. Watson, C. J., in Dock, W., and Snapper, I., eds., *Advances in Internal Medicine*, 1954, v6, 235.

3. Shemin, D., and Russell, C. S., *J. Am. Chem. Soc.*, 1953, v75, 4873.

4. Granick, S., *Science*, 1954, v120, 1105.

5. Vahlquist, B., *Z. physiol. Chem.*, 1939, v259, 213.

6. Granick, S., and Gilder, H., *Adv. in Enzymology*, 1947, v7, 305.

7. Watson, C. J., Schwartz, S., and Hawkinson, V., *J. Biol. Chem.*, 1945, v157, 345.

8. Bogorad, L., and Granick, S., *Proc. Nat. Acad. Sci.*, 1953, v39, 1176.

9. Falk, J. E., Dresel, E. J. B., and Rimington, C., *Nature*, 1953, v172, 292.

10. London, I. M., West, R., Shemin, D., and Rittenberg, D., *J. Biol. Chem.*, 1950, v184, 351.

11. Gray, C. H., Neuberger, A., and Sneath, P. H. A., *Biochem. J.*, 1950, v47, 87.

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Effect of 2-Acetylamino-1,3,4-Thiadiazole-5-Sulfonamide (Diamox) on Sodium, Potassium, Bicarbonate and Buffer Content of Saliva.* (21561)

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The carbonic anhydrase inhibitor, 2-acetylamino-1, 3, 4-thiadiazole-5-sulfonamide (Diamox), has been used to promote diuresis in patients with congestive heart failure since 1952(1). The administration of this compound to such patients is followed by the excretion of an alkaline urine with a high sodium, potassium and bicarbonate content. The loss of fixed base via the urine is due to the inhibition of carbonic anhydrase activity in the kidneys interfering thereby with the production of hydrogen and bicarbonate ions and with the tubular reabsorption of sodium. Since the salivary glands contain an even higher titer of carbonic anhydrase than do the kidneys(2), it was postulated that the inhibition of this enzyme would alter the electrolyte composition of saliva in a manner similar to metabolic acidosis. Accordingly, the present study was devised to determine the effect of 2-acetylamino-1, 3, 4-thiadiazole-5-sulfonamide on saliva sodium, potassium, bicarbonate and buffer capacity in both normotensive and hypertensive patients and to compare these changes with those occurring in patients with metabolic acidosis.

Materials and methods. The effect of 2-acetylamino-1, 3, 4-thiadiazole-5-sulfonamide on the aforementioned salivary electrolytes was determined in 5 normotensive patients and 6 patients with essential hypertension. Base line values for saliva sodium, potassium, bicarbonate and buffer capacity were determined for each patient for from five to seven days prior to the administration of Diamox. The carbonic anhydrase inhibitor was then given orally in divided doses in amounts ranging from 0.25 to 1.5 g per day for a minimum of 2 weeks. Samples of paraffin-stimulated saliva were obtained daily from each patient before breakfast and before brushing the teeth. The paraffin blocks were free of detectable amounts of sodium and potassium. The samples were collected under oil for a period of exactly 16 minutes. They were then centrifuged and the supernatant fluid tested for bicarbonate, sodium, potassium and buffer capacity. Bicarbonate was determined with a Van Slyke blood gas apparatus by the method of Van Slyke and Neill(3). Sodium and potassium were assayed with a Perkin-Elmer Model 52-A Flame Photometer using the internal standard technic(4,5). Buffer capacity was measured by the method of Dreizen, Mann, Cline and Spies(6). *Metabolic acidosis* was induced in 5 arbitrarily selected normotensive subjects by the daily oral administration of from 3 to 9 g of ammonium chloride given in divided doses. Paraffin-stimulated saliva samples were obtained daily from each patient and tested quantitatively for bicarbonate, sodium, potassium and buffer capacity by the methods described above.

Observations. Mean changes in saliva sodium, potassium and bicarbonate concentrations in 5 normotensive and 6 hypertensive

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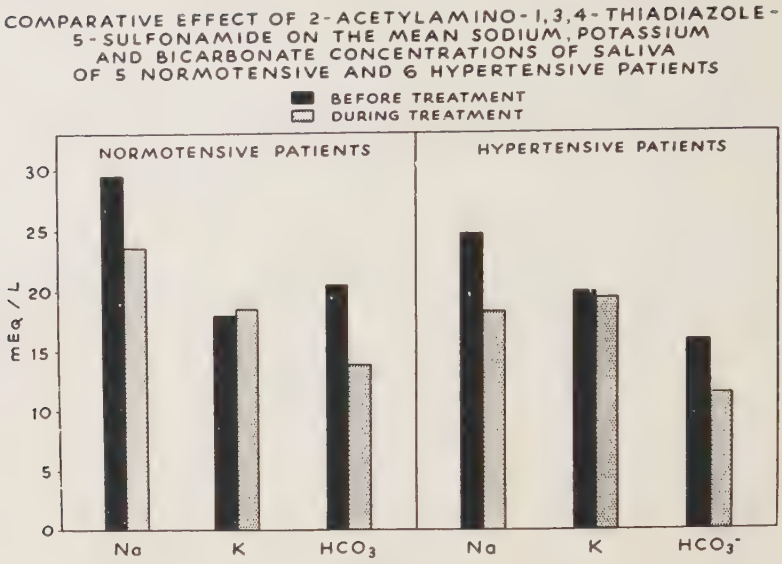

FIG. 1.

patients during the period of 2-acetylamino-1, 3, 4-thiadiazole-5-sulfonamide intake are shown in Fig. 1. The direction of change was identical in each member of each group. Quantitatively, the mean sodium level decreased from 29.6 mEq/L to 23.6 mEq/L in the normotensive group and from 24.7 mEq/L to 18.2 mEq/L in the hypertensive group. The mean bicarbonate content decreased from 20.4 mEq/L to 13.8 mEq/L in the normotensive group and from 15.7 mEq/L to 11.3 mEq/L in the hypertensive group. There was no appreciable change in the mean potassium concentration in either group.

The reductions in saliva sodium and bicarbonate encountered during the administration of the carbonic anhydrase inhibitor were accompanied by a notable decrease in buffer capacity and rate of flow.

Fig. 2 illustrates the effects of acidosis induced by the daily administration of ammonium chloride on saliva sodium, potassium, bicarbonate and buffer capacity in normotensive persons. The findings are representative of those noted in each of the five subjects so treated. In each, there was a pronounced reduction in saliva sodium and bicarbonate reflected by a corresponding diminution in buffer capacity.

Discussion. In recent years, considerable progress has been made in elucidating the factors which control the electrolyte composition of human saliva. Studies performed in this laboratory have shown that rate of flow, adrenal cortical activity and systemic blood pressure may each influence the concentrations of those salivary electrolytes which predominate in the extracellular fluid compartments(7-9). The present findings indicate that alterations in the acid base balance of the blood which are characterized by a diminution in the alkaline reserve are accompanied by a measurable reduction in the sodium, bicarbonate and buffer content of saliva. The action of the carbonic anhydrase inhibitor in reducing the alkalinity of saliva is consistent with the changes reported for urine which result in a lowering of the alkaline reserve. They also conform with the observations of Rapp(10) that the addition of a carbonic anhydrase inhibitor to saliva interferes with the liberation of carbon dioxide thereby preventing the alkalinization of this secretion.

Summary and conclusions. 1. Daily administration of 2-acetylamino-1, 3, 4-thiadiazole-5-sulfonamide provokes a pronounced decrease in sodium, bicarbonate and buffer content of paraffin-stimulated human saliva in both normotensive and hypertensive patients. Potassium level of saliva was not affected.

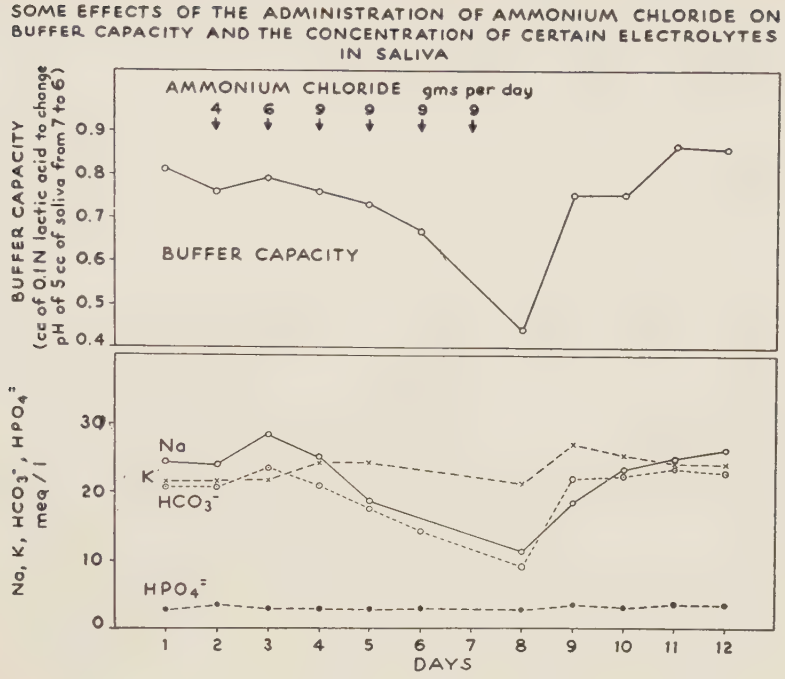

FIG. 2.

2. Changes in saliva sodium, potassium, bicarbonate and buffer content which followed administration of carbonic anhydrase inhibitor were similar to those observed in normotensive persons in whom metabolic acidosis was induced with ammonium chloride.

1. Friedberg, C. K., Halpern, M., and Taymor, R., *J. Clin. Invest.,* 1952, v31, 1074.

2. Hashi, T., *Igaku To Seibutsugku,* 1950, v17, 339.

3. Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.,* 1924, v61, 523.

4. Berry, J. W., Chappel, D. G., and Barnes, R. B., *Ind. Eng. Chem. Anal. Ed.,* 1946, v18, 19.

5. Bills, C. E., McDonald, F. G., Niedermeier, W., and Schwartz, M. C., *Anal. Chem.,* 1949, v21, 1076.

6. Dreizen, S., Mann, A. W., Cline, J. K., and Spies, T. D., *J. D. Res.,* 1946, v25, 213.

7. Dreizen, S., Reed, A. I., Niedermeier, W., and Spies, T. D., *ibid.,* 1953, v32, 497.

8. Niedermeier, W., Dreizen, S., Stone, R. E., and Spies, T. D., *Oral Surg., Oral Med., and Oral Path.,* in press.

9. Dreizen, S., Niedermeier, W., Reed, A. I., and Spies, T. D., *J. D. Res.,* 1952, v31, 271.

10. Rapp, G. W., *J. Am. Dent. Assn.,* 1946, v33, 191.

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Reserpine (Serpasil) Effects on Epileptic Monkeys.* (21562)

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Recent studies have indicated that reserpine, a crystalline alkaloid derived from *Rauwolfia serpentina*, may profoundly affect central nervous system function(1). The pharmacologic effects described suggested the desirability of testing this drug on epileptic monkeys.

Method. Chronic experimental epilepsy was produced in 6 monkeys by the application of alumina cream to a cerebral hemisphere (2). Reserpine (Serpasil)‡ was administered intravenously (1 mg/kg) and the subsequent behavioral, electroencephalographic and epileptic status of treated monkeys were noted for periods up to 4 hours. The character, duration and frequency of spontaneous convulsions, the clinical responses of treated epileptic monkeys to stick prodding for 1 minute and the effects of intramuscular injections of 16 mg/kg or less of 10% pentamethylenetetrazole (Metrazol®) were determined.

Results. Behavioral changes were usually evident within one-half hour following intravenous injection of 1 mg/kg reserpine. Aggressive, hostile, active monkeys became quieter, less resistant, tremulous, sialorrheic, lethargic and less aware of external stimuli. Maximal effects were apparent approximately 2 hours after injection; by this time treated monkeys were semi-stuporous, making little effort to alter their position. Clinical effects of reserpine treatment gradually receded thereafter, monkeys generally returning to pretreatment status within 24 hours.

In general, little change was observed in the electroencephalogram of reserpine treated monkeys that could be ascribed to a specific drug effect (Fig. 1). In some tests, increased amounts of diffusely distributed 5 to 8 per second waves of moderate amplitude were apparent, particularly during periods of lethargy.

Epileptic monkeys which responded to stick prodding for 1 minute with motor convulsions during pre-treatment control periods, frequently were refractory or non-responsive to such stimulation at the time of maximal reserpine effect (2 hours after intravenous injection). However, intramuscular injection of small doses of Metrazol® (16 mg/kg or less) at this time still proved capable of precipitating typical focal motor seizures.

Discussion. In general, the behavioral responses of epileptic monkeys to reserpine treatment resembled those of similarly treated normal monkeys. Particularly striking was the severe degree of sialorrhea, and diffuse, coarse tremors exhibited by some treated epileptic monkeys.

Although a blocking or inhibiting effect to activation of clinical convulsions by the stick prodding technic was noted in some reserpine treated epileptic monkeys, intramuscular injection of small doses of Metrazol® still proved highly effective in provoking typical focal Jacksonian motor seizures in these animals. The lack of response to stick prodding under the circumstances may be explained by the failure of "external" stimulation (prodding) to break through a reserpine induced barrier, for treated monkeys usually showed much less evidence of agitation, physical and emotional stress than similarly tested normals. In this respect our results resembled those cited by Schneider and Earl(1), who stated that reserpine did not prevent chemically induced seizures in animals, whereas a definite antagonism against audiogenic seizures was noted. The recently reported findings of Schneider(3) would also appear to be similar in nature, since it was stated that cats prepared so as to develop sham rage showed little or no reaction when mechanically stimulated ½ to 1 hour after reserpine treatment.

Summary. 1. Intravenous reserpine treat-

* Aided in part by Grant NIH, USPHS. Presented before Eastern Association of Electroencephalographers, Dec. 12, 1954.

† St. Vincent's Hospital, N. Y. City.

‡ Supplied by Ciba.

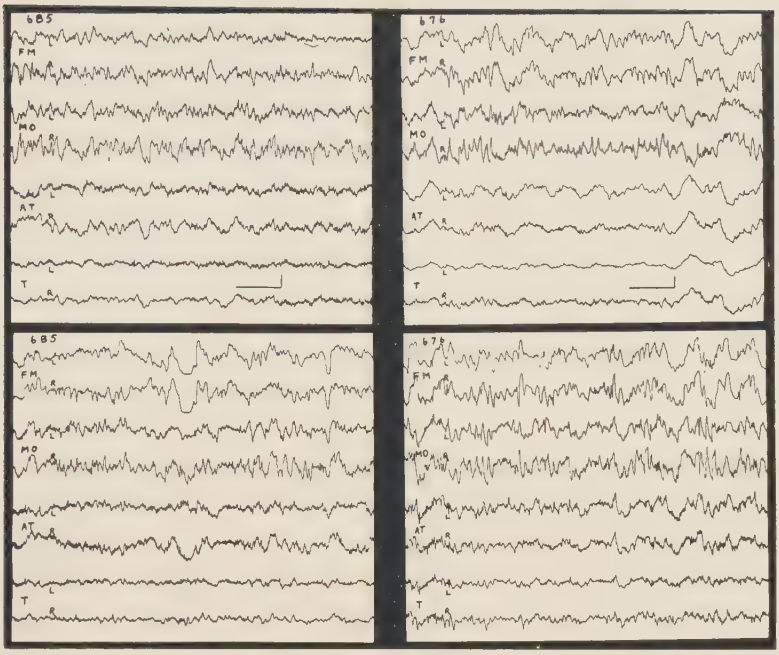

FIG. 1. Electroencephalograms of 2 epileptic monkeys before (top) and 2 hours after (bottom) intravenous reserpine treatment. F = frontal, M = motor, O = occipital, AT = anterior temporal, T = temporal, L = left, R = right. Calibration: 1 sec. (horizontal) and 50 microvolts (vertical).

ment produced behavioral changes in epileptic monkeys resembling those described for similarly treated normals; sialorrhea and tremors were noteworthy in the treated epileptic group. 2. Electroencephalograms of treated epileptic monkeys usually showed little change; 5 to 8/second wave activity of moderate amplitude sometimes was more prominent during lethargic phases. 3. Epileptic monkeys which were more refractory to clinical seizure activation by stick prodding at the height of the reserpine effect, remained highly responsive to activation by small doses of intramuscular Metrazol.®

1. Schneider, J. A., and Earl, A. E., *Neurology,* 1954, v4, 657.

2. Kopeloff, L. M., Chusid, J. G., and Kopeloff, N., *ibid.,* 1954, v4, 218.

3. Schneider, J. A., *Bull. for Med. Research,* 1954, v9, 10.

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Further Purification and Biological Action of Follicle Stimulating Hormone from Sheep Pituitary Glands.* (21563)

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A simplified procedure was reported previously(1) for obtaining a pituitary follicle stimulating hormone preparation of high biological purity. This preparation contained very little luteinizing hormone activity and was free of adrenotrophic, lactogenic and thyrotrophic activities at the levels tested. The essential steps of this procedure are extraction of dry sheep pituitary glands with saturated sodium chloride solution at 70°C, recovery of the follicle stimulating hormone from the extract in the pH 2.5 soluble fraction, removal of salt by dialysis, and concentration of the hormone from the dialyzed fraction by use of an anion exchange resin. This report presents results on further purification of this preparation by use of a cation exchange resin, and data from the study of the biological action of the final product in normal and in hypophysectomized male and female rats.

Materials and methods. The cation exchange resin XE-97 (Rohm and Haas) was activated by treating 20 g with 500 ml of 1 N NaOH for 15 minutes after which the resin was washed free of NaOH with liberal amounts of water by repeated suspension and decantation. This procedure was repeated with 500 ml of 1 N HCl and then again with 1 N NaOH. When the washing was complete after the last treatment with NaOH, the resin was suspended in water and adjusted to pH 6 with 1 N acetic acid. A column of resin of the desired height (usually 30 cm) was formed by pouring the proper amount of suspended resin into a 50 ml burette and allowing it to stand overnight. A circle of No. 576 filter paper (Schleicher and Schuell Co.) was placed on the surface of the column to prevent disturbance of the resin on addition of the sample.

Follicle stimulating hormone (FSH) preparations that had been concentrated by use of the anion resin and dried by lyophilization were dissolved (100 to 300 mg) in 2 to 3 ml of the pH 6 acetate washings obtained during the preparation of the column. This solution of the FSH was passed into the column and then washed with 2 to 3 ml of the acetate washings. The column was developed by adding the acetate washings to a height of 15 cm above the surface of the column and maintaining the liquid at essentially this height during development. Fractions ranging in volume from 3 to 6 ml were collected. Each fraction was assayed for FSH activity in normal female rats. Fractions containing activity were dialyzed and lyophilized to obtain the FSH in dry form. Samples of these fractions were analyzed for nitrogen by the method of Johnson(2) and on the basis of results obtained, the protein content (N x 6.25) of the dry FSH preparations was calculated. This FSH preparation was further purified by use of a column of the cation exchange resin XE-97 adjusted to pH 5. The dried preparation from the pH 6 column was dissolved in the pH 5 acetate washings and passed into the column in the same way as described above. The column was developed first with pH 5 acetate washings which removed 33% of the protein added to the column. This protein was free of gonadotrophic activity. The pH 5 acetate solution was then removed from the column and replaced by 1 M acetate buffer of pH 6. One fraction obtained by use of this buffer contained a major part of the FSH activity added to the column.

Biological assay. The preparations were assayed for gonadotrophic activity in 21-day-old normal female rats of Holtzman-Rolfsmeyer strain. The fractions were adjusted with 0.9% saline solution so that each rat received the proper gram equivalent (g eq.) of dry pituitary tissue in 9 injections of 0.5 ml

* This investigation was supported in part by research grant G-2154(C4) from the National Institutes of Health and by a grant from funds supplied by the Wisconsin Alumni Research Foundation.

TABLE I. Fractionation of FSH by Use of Cation Exchange Resin at pH 6.

No. of fraction*	Vol, ml	Time, min.	Assay		Protein	
			Dose, mg eq.	Wt ovaries, mg	mg/frac.	mg/g eq.
5F-1	6.6	22	1000	11 (1)†		
2	4.7	16	"	18 (3)	18.85	.142
3	5.7	19	"	57 (3)	58.07	.435
4	5.6	20	"	14 (3)	14.49	.110
5	5.4	17	"	14 (3)	12.83	.096
6	5.6	23	"	15 (1)	7.00	
7	5.8	32	"	15 (1)	4.53	
8	5.7	76	"	18 (1)	5.70	

* These fractions were obtained by passing S88A(A-4)FSH (175.5 mg protein equivalent to 133 g of dry pituitary) through a 30 cm column of cation exchange resin XE-97 adjusted to pH 6. When S88A(A-4) was assayed using a total dose of 500 mg eq. the avg wt of the ovaries obtained from 6 normal rats was 56 mg.

† Figures indicate No. of normal rats used for assays.

each. The first injection was made on the afternoon of the first day followed by injections on the morning and afternoon of each of the next 4 days. Autopsy was performed on the morning following the last injection. The ovaries were removed, dissected free of other tissues, weighed and examined by strong transmitted light for the presence of follicles and corpora lutea. Assays were also made using male and female rats which were hypophysectomized when 27 days of age. Injections were started when the rats were 29 days old and were made on the morning and afternoon of each day for 10 days with autopsy on the morning of the 11th day. Ovaries and testes were removed, dissected free of other tissues, weighed and the ovaries examined for the presence of corpora lutea and follicles. The tissues were fixed in Bouin's solution and subsequently prepared for microscopic study.

Results and discussion. The results given in Table I show the volumes of the fractions in relation to the time intervals required for their collection on passing a concentrated solution of FSH (in this case S88A(A-4)) through a 30 cm column of cation exchange resin XE-97 adjusted to pH 6.0. Results of the assay of these fractions, using a total dose of 1 g eq., show that a large part of the FSH activity was recovered in fraction 3 which contained nitrogen equivalent to 33% of the protein added to the column. Approximately 50% of the FSH activity was recovered in fraction 3 (see column 5 and footnote 1 to Table I). The fractions other than 3 contained little activity as shown by assays in which a total dose of 1 g eq. was used. Results given in Tables III and IV, however, show that there was considerable variation in the recovery of FSH activity from different columns and that in some cases the major part of the activity was recovered (Table IV). It is necessary for the FSH to be dissolved in sufficiently concentrated acetate buffer to adjust the protein in the concentrated hormone solution to pH 6 so as to obtain a reasonable rate of flow and high recovery. The low recoveries obtained in the early experiments are presumed to be due, therefore, to the solution not being properly adjusted to pH 6. Average recovery of protein with the FSH fractions from 11 preparations fractionated by use of the column was 41% (Tables III and IV). The protein added to the columns was not quantitatively recovered in the fractions collected by the above procedure.

The pigment present in the FSH preparations obtained by use of the anion resin migrated at a much slower rate than did the FSH. This made it possible to separate the pigment from the hormone. This pigment turned from brown to pink on reduction with sodium hydrosulfite and the reduced pigment showed increased absorption over the oxidized form at 415, 520 and 550 mμ. It also functioned in the succinoxidase system as indicated by oxygen uptake when liver homogenate was used as the source of the enzymes. These results indicate that the pigment separated from the FSH by the cation resin is cytochrome c.

When the column was made of cation resin

TABLE II. Fractionation of FSH by Use of Cation Exchange Resin at pH 5.

Active fraction			Assay	
Exp.*	Protein, mg	% protein added to column	Protein, mg/g eq.	Dose, g eq.	Wt ovaries, mg
22	.74	11	.074	1	45
23	7.63	32	.254	1	76
24	4.75	23	.158	1	38

* FSH20F3 obtained from a column of pH 6 cation resin was used for these experiments. The protein content of this preparation was 0.69 mg per g of dry pituitary tissue and 1 g eq. produced ovaries in normal rats which had an avg wt of 73 mg.

adjusted to pH 5 and the preparation from the pH 6 column was added, the FSH was retained and additional inactive protein filtered through the column. The FSH was removed from this column by further development with pH 6 acetate buffer. The fractions in which the hormone was recovered were variable as to protein content and FSH activity (Table II). These results indicate that a major part of the protein is separated from the FSH activity by this procedure. The procedure is in the process of development, however, and further work is required to determine the optimum conditions for recovering the FSH in a minimum amount of protein. This preparation has not been assayed in hypophysectomized rats and as a result its biological action in the rat has not been compared with that of the FSH preparation from the pH 6 column. Present results do not warrant a conclusion as to whether the mechanism of the separations with the cation resin is one consisting only of filtration of the hormone or whether both filtration and exchange are involved.

Biological action of FSH preparations obtained from cation resin at pH 6. FSH preparations obtained by use of the anion exchange resin XE-59(1) were assayed in normal female rats (Tables III and IV). Results of assays at the 1 g eq. level show that approximately 50% of the activity of these preparations (88, 765 and 1068, Table III) was recovered in the corresponding fractions obtained from the column of cation resin. The ovaries were found to contain only follicles on macroscopic examination at autopsy.

Total doses of 10 g eq. of each of the above 3 FSH preparations from the column of cation resin were given to 29-day-old hypophysectomized female and male rats for 10 days. The results of these assays are given on the right side of Table III. The average weight of the ovaries was 122 mg and they contained only follicles as indicated by macroscopic observation. The uteri were highly distended with fluid. The average weights of the testes, seminal vesicles and prostates were, respectively, 758, 4.9, and 8.3 mg as compared with 141, 5.0, and 6.3 mg for these tissues from uninjected control male rats. These data show that there was an average increase of only 2 mg in the weight of the prostate and none for the seminal vesicle whereas there was a large increase of 617 mg in the weight of the testes.

Microscopic examination of the ovaries of the hypophysectomized rats receiving the most highly purified preparations did not reveal the presence of corpora lutea or lutein tissue. In general the thecal and interstitial tissues of the ovaries did not show much evidence of secretory activity, although there were a few follicles in some ovaries in which there were isolated small areas of thecal cell hypertrophy. The interstitial cells of the testes were involuted and unstimulated. The tubules, however, were relatively very active when compared with hypophysectomized-untreated rats. A few isolated patches of spermatids were observed in some segments of the tubules, but spermatozoa were not seen. The above results indicate that these FSH preparations are of high biological purity in that they contain very litle luteinizing hormone activity.

Preparations 8286, 849 and 789 were on the average about twice as active at the 1 g eq. level as preparations 88, 765 and 1068 (Table III). This was also true for the corresponding F3 fractions obtained from the column of cation resin. This difference in activity does not appear to be a result of using different lots of dry pituitary for making these preparations. Factors that influence the yield of FSH activity are under study and the results may provide information for a possible explanation of the difference in the activity of the two groups of preparations. The average

TABLE III. Results Showing Biological Action in Normal and Hypophysectomized Rats of Follicle Stimulating Hormone Preparations Obtained by Use of Cation Resin.

Preparation*		Assay—Normal rats					Hypophysectomized rats					
										Wt of glands (mg)		
No.	Fraction soluble at pH	Protein, mg/g eq.	Dose, g eq.	Ov. wt, mg	FSH prep. from column	Protein, mg/g eq.	Dose, g eq.	Ov. wt, mg	Dose, g eq.	Ovaries	Testes	S.V.	Ventral prostate
88	2.5	1.32	.5	56 (6)	88- 5F3	.44	.5	36 (3)	10	125 (2)	786 (3)	4.5 (3)	6.1 (3)
			1.0	83 (3)			1.0	46 (6)					
							2.0	128 (3)					
765	2.5	0.93	1.0	75 (3)	765- 7F3	.35	.5	23 (3)	10	106 (3)	716 (4)	4.6 (4)	8.9 (4)
			2.0	118 (3)			1.0	38 (3)					
1068	2.5	0.60	0.5	22 (3)	1068- 8F3	.27	1.0	37 (3)	10	135 (3)	787 (3)	5.7 (3)	9.7 (3)
			1.0	49 (3)			2.0	60 (3)					
			2.0	100 (3)									
	Avg	0.951	1.0	57 (9)		.35	1.0	42(12)	10	122 (8)	758(10)	4.9(10)	8.3(10)
8286	2.5	1.23	.5	106 (3)	8286- 9F3	.60	.5	47 (3)	5	89 (3)	977 (5)	5.4 (5)	10.1 (5)
			1.0	117 (3)			1.0	93 (3)					
849	2.5	1.10	.5	61 (3)	849- 4F3	.40	.5	47 (6)	5	171 (3)	897 (5)	5.5 (5)	7.5 (5)
							1.0	113 (3)					
789	2.5	1.03	.5	53 (3)	789-17F3	.26	.5	34 (3)	5		726 (3)	6.1 (3)	8.4 (3)
							1.0	105 (3)					
	Avg	1.12	.5	73 (9)		.42	.5	44(12)	5	130 (6)	888(13)	5.6(13)	8.7(13)
			1.0	117 (3)			1.0	104 (9)					
Hypophysectomized rats—uninjected controls										6.5 (5)	141(12)	5.0(12)	6.3(12)

* These preparations were obtained by extracting dry sheep pituitary tissue with saturated sodium chloride solution at 70°C, and recovery of the activity from the 2.5 soluble fractions with anion exchange resin.

TABLE IV. Biological Action in Normal Rats of Follicle Stimulating Hormone Preparations Obtained by Use of Cation Resin.

Preparation*		Assay—Normal rats						
No.	Fraction soluble at pH	Protein, mg/g eq.	Dose, g eq.	Ov. wt, mg	FSH prep. from column	Protein, mg/g eq.	Dose, g eq.	Ov. wt, mg
139	2.5	1.07	.5	92 (3)	139-18F3	.5	.5	80 (6)
138	2.5	1.20	.25	42 (3)	138-15F3	.69	.5	113 (3)
			.5	122 (6)			1.0	131 (3)
136–1	2.5	.93	.5	107 (3)	136-1-13F3	.46	.5	100 (3)
	Avg	1.07	.5	111(12)		.56	.5	93(12)
136–2	3.0	1.97	.5	150 (3)	136-2-14F3	.59	.5	101 (3)
134	3.0	2.70	.5	129 (3)	134-12F3	1.17	.25	41 (3)
							.5	62 (3)
	Avg	2.33	.5	140 (6)		.88	.5	82 (6)

* These preparations were made by extracting dry sheep pituitary tissue with 0.9 saturated sodium chloride solution at pH 6 and 70°C, and recovery of the activity from the pH 2.5 or pH 3.0 soluble fractions with anion exchange resin.

weights of the seminal vesicles and prostates from the hypophysectomized rats given 5 g eq. of these preparations were slightly greater than those for control animals. Microscopically the ovaries and testes from the assay of these more active preparations at the 5 g eq. level were essentially the same as those of the less active preparations at the 10 g eq. level.

The preparations given in Table IV were made by extracting dry pituitary tissue with 90% saturated sodium chloride solution at pH 6. As indicated some of these preparations were obtained from the pH 2.5 soluble fraction of the extracts whereas others were prepared from the pH 3 soluble fraction of the 90% salt extract. The passage of these FSH preparations through the column of cation resin at pH 6 separated the major part of the FSH activity into a single fraction which contained on the average 44% of the total protein added to the column. On the basis of assays using normal female rats the FSH preparations (Table IV) obtained by use of 90% saturated sodium chloride show greater activity than do those (Table III) which were prepared by extracting with saturated salt solution.

The presence of carbohydrate in the FSH preparations from the column of pH 6 resin was indicated by a positive Molisch test. Five of these preparations were analyzed by the carbazole method of Gurin and Hood(4) as modified by Holzman, MacAllister and Niemann(5) and were found to contain on the average 9.5% carbohydrate expressed as glucose. The preparation contains hexosamine as shown by the method of Dische and Borenfreund(6). These results indicate that this FSH preparation has a much greater content of carbohydrate than the 1.23% hexose and 1.51% hexosamine reported by Li and Pedersen(3) for their preparation. The high solubility of our preparation (100 g eq. is soluble in 1 ml water) may be due in part to its high carbohydrate content. This FSH preparation obtained from the pH 6 resin contains inert protein which was separated recently by use of a column of the cation resin adjusted to pH 5 (Table II). It is possible that part of the carbohydrate may be associated with this inert protein. In the light of these results the high carbohydrate value given above cannot be properly evaluated until further information is available.

It is of interest to compare the biological action of the FSH preparation reported here (Table III) with the action of pituitary FSH preparations reported by Li *et al.*(7,3), and Van Dyke, P'an and Shedlovsky(8). Steelman *et al.*(9) prepared FSH from an acid acetone residue of hog pituitary glands and reported its biological action as similar to the preparations of Van Dyke and Li. Van Dyke, P'an and Shedlovsky assayed their FSH preparation from hog pituitary glands and a sample of purified sheep pituitary FSH sup-

plied by Dr. Li, by giving a total dose of 10 mg of these preparations to hypophysectomized male rats for 4 days beginning 1 day after hypophysectomy. The weights of the testes and prostate of the treated animals were approximately 3 times the weight of the glands from the control animals. The seminal vesicles were slightly increased in weight over those of controls. On the basis of these results, Van Dyke *et al.* concluded that these preparations contained significant amounts of luteinizing hormone activity. They produced only about half the increase in the weight of the testes and much more increase in the weight of the prostate than did the preparation reported in Table III which was given to hypophysectomized rats for 10 days. After this prolonged treatment the testes were increased 617 mg over those from uninjected control rats and there was very little increase in the weight of the ventral prostate. These results and also those from the microscopic study of the testes and the ovaries of the hypophysectomized rats indicate that the FSH preparation reported here is of high biological purity.

Summary. The FSH preparation obtained previously from a dialyzed salt extract of sheep pituitary glands by use of anion exchange resin was purified extensively by use of columns of cation exchange resin adjusted to pH 6 and pH 5. Results obtained on assay of the preparation from the pH 6 resin in hypophysectomized male and female rats indicate that this FSH preparation is of high biological purity in that it contains very little luteinizing hormone activity.

1. McShan, W. H., Meyer, R. K., and Kagawa, C. M., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 393.

2. Johnson, M. J., *J. Biol. Chem.,* 1941, v137, 575.

3. Li, C. H., and Pedersen, K. O., *J. Gen. Physiol.,* 1952, v35, 629.

4. Gurin, S., and Hood, D. B., *J. Biol. Chem.,* 1939, v131, 211.

5. Holzman, G., MacAllister, R. V., and Niemann, C., *ibid.,* 1947, v171, 27.

6. Dische, Z., and Borenfreund, E., *ibid.,* 1950, v184, 517.

7. Li, C. H., Simpson, M. E., and Evans, H. M., *Science,* 1949, v109, 445.

8. Van Dyke, H. B., P'an, S. Y., and Shedlovsky, T., *Endocrinology,* 1950, v46, 563.

9. Steelman, S. L., Lamont, W. A., Dittman, W. A., and Hawrylewicz, E. J., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 645.

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Effect of W^v Locus in Mouse on Differential Excretion of Isomers of Several Amino Acids.* (21564)

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The D and L isomers of certain amino acids have been shown by Rose(1,2), Du Vigneaud (3), and later investigators(4) to be equally effective, or nearly so, in promoting growth in the rat and mouse.† Only the L-isomers of these amino acids are effective in man, dog (5), rabbit(6), domestic fowl(5,7), and some other animals. In the work to be described it has been found that in mice containing one mutant allele the L-forms only of these amino

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† For those amino acids where there has been a lack of agreement between investigators on growth promoting abilities of the D isomers, Armstrong(18) has recently provided an explanation. He has found in experiments with phenylalanine that where less growth was obtained by feeding the D isomer than by feeding the L isomer, the actual intake of the D form was less, and that when the intake of the D form was controlled, growth was equal to that produced by the L-form.

acids are utilized, while mice containing the normal allele also utilize the D-forms.

The mice in which this observation has been made are those containing the W^v allele in the genotype of the inbred strain C57Black (C57BL) as described by E. S. Russell (8,9, 10). The W, W^v, and w alleles in the mouse are of special interest because the six possible genotypes differ in a regular manner in what appear to be unrelated characters. Whereas the W allele has a dominant effect on pigmentation only, W^v is partially dominant in its effects on pigmentation, size, and number of erythrocytes. The differences in pigmentation suggest a possible connection with the aromatic amino acids concerned with melanin formation and for this reason amino acid metabolism in these mice was studied. The possibility that a disturbed amino acid metabolism might also be concerned with the macrocytic anemia of these mice was also suggested by the observations of Svenseid, Burton and Bethell(11) on pernicious anemia patients in relapse, and of Levine, Marples, and Gordon(12) on the anemia of premature infants. The study of amino acid metabolism to be described, does not in fact further our understanding of either the pigmentation or the anemia in these mice. The new observations do, however, add a curious character to those already known to be affected by the W and W^v loci. This may perhaps be of general interest for problems concerning the metabolism of D and L amino acids.

Materials and methods. The animals were from matings of heterozygous sibs (W^vw x W^vw) from a line back-crossed to C57BL for 21 generations. C57BL and inbred Bagg albino (Balb) mice were used as additional controls in the feeding experiments. Prior to the feeding experiments all animals had been on conventional Rockland pellet diet with water *ad lib.* To test whether the W^v gene and not the entire genotype was responsible for the differences observed in excretion of the D and L forms, mice were obtained from E. S. Russell in which the W^v gene was on a different genetic background. Urine was collected in a glass apparatus designed especially for this purpose; it was a modification of a metabolism cage. A glass cylinder with its bottom removed was placed over a large funnel. Mice were supported on stainless steel screening with a mesh fine enough to prevent the feces from dropping into the urine. Urine from 4 animals was collected individually during 14 hours, food withheld and water given *ad lib.* A few crystals of thymol were added to the urine vial underneath the funnel to prevent bacterial contamination. Unless the urine

TABLE I. Relative Proportion of Phenylalanine Excreted per Unit Creatinine in Sample, in 16 Experiments.

Genotype & strain	Isomer fed						
	D		DL			L	
C57BL	1	2	3	4	5	6	7
W^vW^v	15, 10	22	23, 15	9, 4	14	0, 0	0
W^vw	4	4, 9	10	3	4, 4	0	0, 0
ww	1	2	3	2	1	0	0
C57BL	8		9			10	
++	4, 3		4, 4, 3			2, 2	
	3, 4					2, 2	
C3H	11		12	13			
WW^v	5		3, 3	10			
W^vw	4		—	6			
Ww	1		1	2			
ww	1		1	2			
Balb	14		15			16	
AAbbcc	4, 4, 5		3, 3			1, 1	
			3, 3			1, 1	

TABLE II. Relative Proportion of Tryptophan Excreted per Unit Creatinine in Sample, in 13 Experiments.

Genotype & strain	Isomer fed					
	D		DL		L	
C57BL	1	2	3	4	5	6
W^vW^v	17, 18	25, 29	15, 13	16, 14	2, 2	4
W^vw	14	16	15	10	5	2, 1
Ww	6	9	6	5	2	1
C57BL	7		8		9	
++	6, 8, 9		7, 5		1, 2	
			7, 6		1, 2	
C3H			10	11		
WW^v			11	12		
W^vw			—	7		
Ww			2	—		
ww			3	5		
Balb	12				13	
AAbbcc	3, 1, 3				2, 2	
					3, 5	

TABLE III. Relative Proportion of Methionine Excreted per Unit Creatinine in Sample, 14 Experiments.

Genotype & strain	Isomer fed					
	D		DL		L	
C57BL	1	2	3	4	5	6
W^vW^v	11, 22	12, 9	2, 2	2, 2	0, 0	0
W^vw	3	2	1	1	1	0
ww	0	0	0	0	0	0
C57BL	7		8		9	
++	0, 0		0, 2 3, 2		3, 3	
C3H			10	11		
WW^v			1, 1	1, 1		
W^vw			0	—		
Ww			—	0		
ww			0	0		
Balb	12		13		14	
AAbbcc	1, 1		1, 1, 1		5, 3	

was used immediately it was stored in a deep freeze. The urine sample was applied to filter paper for chromatographic analysis of the amino acids. First, however, a quantitative creatinine determination was performed on each sample. Creatinine was used as a reference substance because it has been found that this constituent is not reabsorbed and shows less individual variation than any other substance studied. Creatinine was determined by a modification of the method of Bonsnes and Taussky(13) using alkaline picric acid and reading colorimetrically at 540 mμ. A standard creatinine curve was prepared for each run and the concentration of urine samples determined from this. Each urine sample from an individual litter was applied to the filter paper so that it represented the same final creatinine concentration. This facilitated measuring the spots developed by ninhydrin later. A 2-dimensional chromatographic method was employed(14,15) using phenol and lutidine and the sheets dipped in 2% ninhydrin(16). For the chromatography of human urine .2% ninhydrin is commonly used; however, Wellington has shown that 2% ninhydrin produces a maximum development of the amino acid spots. Duplicate sheets were run for each sample and all 4 samples collected during a single period were tested chromatographically at the same time on 10 x 10 cm aluminum racks which held 12 sheets. The spots developed by ninhydrin were identified by reference to standard sheets prepared by chromatographing a mixture of 15 known amino acids in known concentrations. These standards were run on duplicate sheets in conjunction with each set of urine samples. Throughout this work an effort was made to chromatograph all urine samples collected over a single period at the same time, so that any variations arising from differences in temperature or humidity would affect those samples equally. The spots on the urine sheets developed in ninhydrin were read in a densitometer and optical density values for the amino acids recorded. A standard curve for each amino acid was prepared relating the known concentration of the amino acid to its densitometer reading. Each curve represented the mean of 6 chromatographic runs of an amino acid mixture of 15 amino acids, each of the 7 concentrations run in duplicate. The optical density readings from the urines were then converted to milligrams amino acid excreted using the standard amino acid curves and this figure then divided by the creatinine concentration of the urine on the filter paper. Where an amino acid solution had been fed, the milligrams excreted for a given creatinine concentration were divided by the milligrams amino acid solution ingested. The final value represents the relative proportion of the amino acid in the ninhydrin-developed spot.

Sample Calculation.

Genotype	Mean optical density	mg in sample	$\frac{\text{mg excreted}}{\text{mg creatinine}}$	$\frac{\text{mg excreted/}}{\text{mg creatinine}}{\text{mg amino acid ingested}}$
W^vW^v ♀	.850	.014	.014/.15 = .093	.093/40 = .0025
W^vW^v ♂	1.080	.018	.018/.15 = .116	.116/74 = .0015
W^vw ♂	.715	.012	.012/.16 = .075	.075/74 = .0010
ww ♀	.350	.004	.004/.15 = .026	.026/80 = .0003

Results. Tables I, II, III, IV, and V give the results of feeding those amino acids whose

TABLE IV. Relative Proportion of Histidine Excreted per Unit Creatinine in Sample, 13 Experiments.

Genotype & strain	Isomer fed					
	D		DL		L	
C57BL	1	2	3	4	5	6
W^vW^v	13, 8	6	18	7, 11	0	0
W^vw	8	5, 5	8, 5	4	0	0
ww	2	2	4	1	0	0
C57BL	7		8		9	
++	3, 0, 0		0, 0 0, 0		0, 0 0, 0	
C3H			10	11		
WW^v			8, 6	8		
W^vw			3	—		
Ww			—	1		
ww			2	1		
Balb	12				13	
AAbbcc	2, 2, 2				0, 0 0, 0	

D and L isomers have been shown to produce equal or nearly-equal growth in the mouse. These amino acids are phenylalanine, tryptophane, methionine, histidine(4) and tyrosine (17). It can be seen that W^vW^v, W^vw and *ww* as well as C57BL and Balb excreted very little if any of the L form as measurable with the method used. This was also true for *ww*, C57BL and Balb when the racemic mixture and the D isomer were fed. The W^vW^v and W^vw on the other hand, show a marked and measurable excretion when the D form and racemic mixture were fed. It can be seen from these tables that the excretion of the W^vw mice was not consistently intermediate between the W^vW^v and the *ww*. These tables also show that similar results are obtained when the gene is on a different genetic background. The effect of increasing the dosage of the W^v produces the same effect on the excretion of the D-forms of these amino acids as it has on other characters affected by these alleles, for example on the number of erythrocytes and intensity of pigmentation.

Tables VI and VII give the results of feeding leucine and threonine, amino acids whose D-forms have *not* been shown to produce appreciable growth in the mouse. The excretion of fed DL and L-leucine is very similar while

TABLE V. Relative Proportion of Tyrosine Excreted per Unit Creatinine in Sample, 10 Experiments.

Genotype & strain	Isomer fed				
	DL			L	
C57BL	1	2	3	4	5
W^vW^v	20	17	26, 31	0, 0	0, 0
W^vw	17	13	10	0	0
ww	0	0	3	0	0
C57BL				6	
++				2, 2 2, 2	
C3H	7	8			
WW^v	26	10			
W^vw	6	—			
Ww	—	2			
ww	3	1			
Balb	9			10	
AAbbcc	5, 5 5, 5			2, 2 2, 2	

TABLE VI. Relative Proportion of Leucine Excreted per Unit Creatinine in Sample, 8 Experiments.

Genotype & strain	Isomer fed			
	DL		L	
C57BL	1	2	3	4
W^vW^v	2	1, 3	1	0
W^vw	1, 2	1	1, 1	0
ww	3	1	1	1
C57BL	5		6	
++	1, 2, 1, 2		2, 2 2, 2	
Balb	7		8	
AAbbcc	1, 1 1, 1		0, 2 1, 0	

TABLE VII. Relative Proportion of Threonine Excreted per Unit Creatinine in Sample, 5 Experiments.

Genotype and strain	Isomer fed		
	D		DL
C57BL	1	2	3
W^vW^v	25, 27	38	39, 25
W^vw	27, 41	36	28
ww	61	44	30
C57BL	4		
++	29		
Balb	5		
AAbbcc	6, 37		

mice fed the DL and D threonine show high excretion of threonine.

A significant feature of the results is their consistency. Within any experiment in which the D isomer was fed W^vW^v showed a higher concentration of excreted amino acid than W^vw and the heterozygote showed a higher concentration than *ww*. There are, however, no available statistical methods which can be applied to this material which will indicate significant differences in excretion concentrations.

To account for the inability of mice with the W^v allele to utilize certain D-amino acids, absence of D-amino acid oxidase might be postulated. It was thought during the early studies on inborn errors that all conditions classified as metabolic errors were due to lack of enzymatic activity(19). However, it has now developed that in some cases, cystinuria, for example(20), the condition has been shown to be due to a variation in renal function (which, incidentally, may also be enzymatic). Modified renal activity may account for the condition observed in W^v mice. A possible difference in intestinal flora should also be considered(21). Which if any of these explanations applies to the present work is still to be determined.

It has not been possible to recognize any physiological or biochemical connection between the characters already known to be affected by the W^v allele; nor is there any apparent connection between any of these characters and the change in amino acids metabolism which is described here. It is, however, noteworthy, that with respect to the utilization of certain D-amino acids the W^v mice differ from other mice, but in this respect resemble a number of other mammals.

Summary. Mice of W^vW^v, WW^v, and W^vw genotypes, unlike other mice, excrete the D-isomers of phenylalanine, tyrosine, tryptophane, methionine and histidine when the D + L isomers of these amino acids are fed. *Ww* and *ww* mice, however, otherwise genetically similar to the above, are able to utilize both the D and L isomers of these amino acids.

1. Rose, W. C., and Womack, M., *J.B.C.,* 1946, v166, 103.

2. ———, *Physiol. Rev.,* 1938, v18, 109.

3. Du Vigneaud, V., Sealock, R., and van Etten, C., *J.B.C.,* 1932, v98, 565.

4. Bauer, C. D., and Berg, C. P., *J. Nutrition,* 1943, v26, 51.

5. Greenberg, D. M., *Proteins and Amino Acids,* Chap. X, Thomas, 1951.

6. Cox, G. J., and Berg, C. P., *J.B.C.,* 1934, v107, 497.

7. Wilkening, M. C., and Schweigert, R., *ibid.,* 1947, v171, 209.

8. Russell, E. S., *Genetics,* 1946, v31, 327.

9. ———, *ibid.,* 1948, v33, 228.

10. Russell, E. S., and Fondal, E. L., *Blood,* 1951, v6, 892.

11. Svenseid, M. E., Burton, I. F., and Bethell, F. H., PROC. SOC. EXP. BIOL. AND MED., 1943, v52, 202.

12. Levine, S. Z., Marples, E., and Gordon, H. H., *J. Clin. Invest.,* 1941, v20, 199.

13. Bonsnes, R. W., and Taussky, H. H., *J.B.C.,* 1945, v158, 581.

14. Dent, C. E., *Biochem. Symp.* No. 3, 1949.

15. Block, R. J., LeStrange, R., and Zweig, G., *Manual of Paper Chromatography,* Academic Press, 1952.

16. Wellington, E. F., *Canad. J. Chem.,* 1952, v30, 581.

17. Martin, G. J., *Biological Antagonism,* Chap. 7, Blakiston, 1951.

18. Armstrong, M. D., *J.B.C.,* 1953, v205, 839.

19. Garrod, A. E., Croonian Lectures, *Lancet,* 1908, v2.

20. Harris, H., *Eugenics Memoirs,* 1953, v37.

21. Haldane, J. B. S., *Biochemistry of Genetics,* p82, George Allen, and Unwin, 1954.

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Erythrocyte Metabolism in Sickle Cell Anemia. (21565)

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Sickle cell anemia is characterized by red blood cells which assume atypical shapes at low oxygen concentration. Sickle hemoglobin differs from normal electrophoretically and by its reduced solubility(1,2) when deoxygenated, the latter probably accounting for the distorted erythrocytes. Itano and Pauling (3,4) have proposed that an inherited abnormality of the single protein, hemoglobin, brings about the sequence of events that results in the disease. There is as yet no direct evidence, however, that the abnormal hemoglobin is a cause of the premature hemolysis of the sickle cell anemia erythrocyte. The red blood cell is a complex unit whose integrity is dependent on an active carbohydrate metabolism(5). It has highly specialized selective permeability properties which undoubtedly are supported by the metabolic energy. It contains several enzymes in significant quantities, such as carbonic anhydrase, catalase and cholinesterase which could play important functional or maintenance roles. It has a unique lipoprotein surface structural material (stroma). An inherited abnormality of one or more of these non-hemoglobin proteins could conceivably result in a decreased life span of the sickle cell.

We have examined a case of sickle cell anemia in some detail in order to determine whether any of the red cell constituents associated with its carbohydrate metabolism have shared in this inherited anomaly.

Methods. Experiments were performed on blood from a typical sickle cell anemia patient; male, negro, age 30. His average hematologic status was as follows: RBC 2.8 million, PCV 29%, WBC 22,000, Hb 8.4 g, retics 11%, serum bilirubin 3.5 mg %, urine urobilinogen 25 mg %. Eighty percent or more of the red cells sickled on gassing with carbon dioxide. Average red cell life span, chromium 51, corrected for elution, 10-20 days. Blood samples were taken over a period of several weeks during which the blood values were stabilized. Following this period the patient had a hemolytic crisis and was transfused. After the transfusion a hemoglobin paper electrophoretic analysis gave the following results: 85% sickle, 14% normal, 1% fetal(6). *Sickle anemia blood* has typically elevated leukocyte and reticulocyte concentrations. These 2 cells have higher and qualitatively different metabolic activities than the mature erythrocyte. For all experiments here reported the sickle erythrocytes were separated by repeated centrifugation in plasma, each time discarding the top layers of blood cells which contained concentrated platelets, leukocytes and reticulocytes. The separated erythrocytes were always incubated in their own plasma. There was no evidence that these manipulations disturbed the metabolism of normal erythrocytes. *Blood* was collected in heparin and used within a few minutes. Glycolytic intermediates were assayed by ion-exchange chromatography(7).

Results. Mature erythrocytes have a predominantly glycolytic metabolism which is independent of oxygen concentration(8,9). In order to evaluate this metabolism in sickle cell anemia, three facets were examined: glucose utilization and lactate formation, the level and kind of carbohydrate intermediates, and, P^{32} uptake. Two aspects of the problem are of interest. First, does the sickle erythrocyte show normal glycolysis when in the biconcave configuration? Second, is its metabolism altered when it assumes the sickle form?

In 6 determinations on blood samples taken on different days the rate of both glucose utilization and of lactate formation was between 30 and 35 mg/100 ml of cells per hour, which falls within the normal range(10). Samples were incubated in air so that the red cells were not sickled.

To obtain most of the cells in the sickle form, it is not sufficient to remove oxygen. The pH must also be lowered. The separated sickle anemia erythrocytes suspended in their own plasma were divided into 2 parts; one gassed with wet 50% CO_2-O_2, and the other

gassed with wet 50% CO_2-N_2, giving a pH of 6.8 in each case. In the oxygen mixture all cells were normal in shape while under N_2-CO_2, 100% of the cells showed various degrees of distortion. The 2 portions were incubated at 38°C for 3 hours. Glucose utilization was approximately the same, but the method had poor reliability due to a marked drop in glycolytic rate with lowering of pH.

The fixation of inorganic P^{32} phosphate in red cell organic phosphates is, in short time intervals, proportional to glucose utilization. Radioassay of P^{32} incorporation was chosen as a more sensitive measure of the carbohydrate metabolism in the above experiment. There was no difference in P^{32} uptake by the sickle cell anemia erythrocyte whether in the normal or sickled configuration. For example, in one experiment, 0.05 ml of inorganic $P^{32}O_4$ was added to 10 ml of an ice-cold 30% sickle erythrocyte suspension. The blood was divided into 2 flasks which were kept in an ice bath while being gassed for 5 minutes respectively with 50% CO_2-N_2 and 50% CO_2-O_2. The 2 portions were incubated with shaking at 38°C and samples taken at 0, 30 and 90 minutes. Sickled and non-sickled cells gave identical P^{32} uptakes. Cts/min: 0 = 1700, 30 = 17,000, 90 = 26,000.

The normal actively glycolysing red cell has a characteristic pattern of carbohydrate intermediate phosphates and adenylates which can be recovered and assayed with anion exchange resins(7). The level of these metabolic intermediates represents a balance between continuous formation and breakdown and when this steady state is disturbed, there results a shift in population. These compounds then provide an index to the amounts and functioning status of the individual glycolytic enzymes. A confirmatory test of metabolic activity is given by the rate of movement of inorganic $P^{32}O_4$ into these metabolites(11).

Twenty ml of 30% separated sickle cells in plasma were incubated for 3 hours at 38°C with a trace of inorganic $P^{32}O_4$. The cells were then washed with saline to remove plasma and unreacted labeled phosphate, extracted with trichloroacetic acid, the TCA removed with ether and the extract chromatographed on Dowex-1-Cl. The resulting spectrum of the red cell phosphorus compounds and the extent of labeling with p^{32} was the same as that obtained with normal red cells (7,11).

It had seemed possible that an inherited defect of some sickle cell metabolic component would lead to premature hemolysis. From the evidence submitted it appears that none of the several enzymes responsible for operation of glycolysis has been sufficiently modified to disturb the metabolism.

A secondary distortion of glycolysis might occur if some obligatory anatomical arrangement of the enzymes were disrupted by the change from normal to sickle shape. The evidence is against such an effect.

There are, of course, other important nonhemoglobin constituents of the red cell which, if abnormal, might lead to a reduced life span, but, by the process of elimination, the present study provides more support for the concept that hemoglobin is the sole responsible agent for the production of anemia.

Summary. Several tests of carbohydrate metabolism in the sickle cell anemia erythrocyte did not disclose any abnormality.

1. Perutz, M. F., and Mitchison, J. M., *Nature,* 1950, v166, 677.

2. Perutz, M. F., Liquori, A. M., and Eirich, F., *Nature,* 1951, v167, 929.

3. Pauling, L., Itano, H. A., Singer, S. J., and Wells, I. C., *Science,* 1949, v110, 543.

4. Itano, H. A., *Science,* 1953, v117, 89.

5. Bartlett, G. R., and Marlow, A. A., *Bull. Scripps Metabolic Clinic,* 1951, v2, No. 5.

6. Courtesy of Dr. A. Motulsky.

7. Bartlett, G. R., Savage, E., Hughes, L., and Marlow, A. A., *J. Appl. Physiol.,* 1953, v6, 51.

8. Bartlett, G. R., and Marlow, A. A., *J. Lab. Clin. Med.,* 1953, v42, 178.

9. ———, *ibid.,* 1953, v42, 188.

10. ———, *J. Appl. Physiol.,* 1953, v6, 335.

11. Bartlett, G. R., *Fed. Proc.,* 1954, v13, 179.

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Recognition of Mouse Ectromelia. (21566)

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Two viruses received from abroad have unexpectedly been found to resemble mouse ectromelia. Both agents were originally considered to have caused human disease of the central nervous system and their behavior in mice inoculated by the methods commonly used in the study of neurotropic or Coxsackie viruses had not suggested their true nature. The present résumé of our experience is reported to call attention to the fact that ectromelia has masqueraded as a neurotropic virus and to alert American workers to the danger of introducing the virus into their mouse colonies. The two agents had been received at different times from widely separated European laboratories. One was sent to us for comparisons with Coxsackie strains, the other as an atypical lymphocytic choriomeningitis virus. A number of tests had been performed with each before their similarity became evident. Marchal's(1) original description of the behavior of infected mice was based on natural infections in a colony. Dermal lesions of the feet and an unexplained high death rate among her stock were the clues that attracted attention. In recent reports(2,3) of 2 domestic outbreaks, unexplained deaths were first noted and later the appearance of swelling and necrosis of the legs. In all cases the colonies were presumably generally infected and there was some difficulty at first in experimentally infecting stock mice which may have been in part immune. Marchal transmitted infection by cohabitation, and by intradermal, intraperitoneal, intravenous, and subcutaneous injections. We have found no published observations of the responses in fully susceptible stock mice following inoculation by the methods used in the study of neurotropic viruses. Schoen found no characteristic pathologic changes in the central nervous system, even in mice inoculated by the intracerebral route(4). Fenner found suckling mice more susceptible than weanling mice, unless their mothers were immune(5).

Results. Weaned 8- to 12-g mice of the Albany standard strain responded to intracerebral injections of both viruses with the violent tetanic convulsions, followed by bladder incontinence and often by death, that are so characteristic of experimental lymphocytic choriomeningitis. The incubation period was 4 to 5 days, shorter than that of lymphocytic choriomeningitis, which has seldom been less than 6 to 7 days in our experience. Intraperitoneal injections induced similar responses in the same length of time in some animals but others that looked sick never developed convulsions. Suckling mice were highly susceptible to infection by both routes. They were depressed and turned a peculiar plum color, especially in the extremities, often as early as the third day. Death was sudden. Convulsions and questionable paralyses were observed in young animals on rare occasions.

Post-mortem examinations of mice of both ages provided the first strong indication that we were dealing with agents different from lymphocytic choriomeningitis or Coxsackie virus infection. The suckling mice were the first to be studied in detail because of their peculiar ante-mortem appearance and because it has become second nature for us to check suckling mice for the gross muscle and fat pad lesions characteristic of Group A and Group B Coxsackie virus infections. Three unexpected and consistent gross lesions were seen. The livers were very pale, the spleens were slightly enlarged and contained hemorrhagic areas, and the upper intestinal tract was congested and edematous. Lymphocytic choriomeningitis has little effect on the gross appearance of these organs, and the Coxsackie viruses of Group B, while they sometimes are associated with pale livers and bloody material in the large intestines, do not affect the spleen. Mouse hepatoencephalitis viruses such as the JHM strain(6) with which we are familiar, produce focal necroses of the liver but it is quite different from the yellow appearance in mice inoculated with the 2 "unknown" viruses.

Since the weaned mice were at first assumed to have involvement of the central nervous

system, little attention was paid to the appearance of their viscera until the lesions in the newborn were recognized to be a constant feature of infection. It was then found that the older mice, especially after intraperitoneal inoculation, had similar lesions in their livers, spleens, and small intestines, and in addition a few showed necrosis of the visceral fat of the kind that follows leakage of pancreatic secretions and a great increase in pleural and peritoneal fluids.

Serologic studies were consistently unhelpful and it proved impossible to produce a satisfactory homologous neutralizing antiserum with either virus in rabbits, guinea pigs, or hamsters. Mouse antisera were not prepared. This decision was based in part on our appreciation that our stock animals have antibodies for mouse hepatoencephalitis virus, some strains of which cause widespread hepatic lesions somewhat similar to those being studied. It is possible that if we had attempted to prepare specific mouse sera by repeated intraperitoneal injections of the two unidentified viruses, we might have discovered earlier that ectromelia was involved, for it seems probable that the large mice would have developed a generalized infection during immunization. Among the known antisera that at one time or another were included in serologic tests and gave no indication of neutralization were those of lymphocytic choriomeningitis, herpes, Theiler's GD VII, MM, Japanese B encephalitis, and various serologic types of Coxsackie Group A antisera. Pooled normal human sera were also inactive. The neutralization tests were done in suckling mice by the intraperitoneal route.

Both viruses tolerated lyophilization, and one was shown to pass a Seitz filter and to be largely inactivated by repeated freezing and thawing. Neither virus was pathogenic for hamsters or guinea pigs, young or old. One strain was inoculated on the chorioallantoic membrane of embryonated eggs. It produced pocks on the membrane, but inclusion bodies were not recognized. Herpes virus was considered until it was found that the unknown virus had no effect on the cornea of a rabbit, and was not neutralized by herpes antiserum.

A visiting European colleague who was familiar with ectromelia did not recognize the disease from the behavior of the suckling or weaned mice but assured us that the appearance of the liver was compatible with that diagnosis. Histologic examination provided strong support. The lesions conformed closely with Schoen's description(4). Schoen considered the changes in the liver and spleen to be the most interesting lesions of ectromelia. He characterized the hepatic lesion as an interstitial necrosis with cytoplasmic inclusions composed of fine, basophilic granules that frequently coalesced into oval or round colonies. Some inclusions, he wrote, were larger and more homogeneous and these were surrounded by clear halos. This describes our own material as well, although we would define the interstitial changes more closely. To us they have seemed noteworthy because of the edema associated with blood sinuses and smaller vessels, a lesion of endothelial elements. It may be of some interest that in our heavily infected immature animals the hepatic inclusions were regularly present although generally amorphous and basophilic, and in mice which died after the briefest incubation periods they were less numerous. The splenic necroses were associated with the central arteries and consisted of relatively large necrotic areas having a concentric or onion skin pattern. In and among the cells were not only nuclear debris but also inclusion bodies.

The agent was further identified by inoculating adult mice into their footpads. Both strains were tested and both behaved identically. Several days after the injection of 0.05 ml of a 10% suspension of infected tissue the foot became red and swollen. The lesion was well developed by the fifth or sixth day and progressed with cracking of the skin and patches of necrosis. A few of these animals developed generalized infection and died. In them, characteristic hepatic lesions were seen. On the eleventh day all the mice were destroyed as a safeguard against accidental infection of other animals. The dermal lesions were consistent with Marchal's description. Liquefaction and blister formation within the corium were found on occasion and in some lesions cytoplasmic inclusion bodies were conspicuous.

TABLE I. Protective Effect of Vaccinia against Infection with the Unknown Viruses.

Challenge virus			
Strain	Route	Response of exp. group	Response of controls
"A"	Footpad	—, —, +, +, ++, ++, ++	11, 3+, 3+
"	Intracer.	5, 5, 5, *S*, *S*, *S*, *S*	5, 5
"	Intraper.	11, *S*, *S*, *S*, *S*, *S*, *S*	6, 7
"B"	Footpad	—, —, —, +, +, ++, ++	3+, 3+, 3+, 3+
"	Intracer.	*S*, *S*, *S*, *S*, *S*, *S*, *S*	7, 7
"	Intraper.	*S*, *S*, *S*, *S*, *S*, *S*, *S*	11, 12

Numerals indicate day of death; *S*, survival; plus signs, the severity of lesions of footpads.

The agent was further identified by challenging mice that had 4 weeks previously been inoculated with a 10% suspension of chorioallantoic membranes infected with a repeatedly passaged strain of a commercial calf lymph preparation of vaccinia virus. The mice were inoculated intracerebrally (0.03 ml) or intraperitoneally (0.01 ml). Groups of convalescent mice were challenged with each unknown. The challenge inoculations were intracerebral, intraperitoneal or intradermal. One challenge virus was used in a 10% suspension of baby mouse carcass and brain, the other in a 1% suspension. The results are shown in Table I. It is evident that a significant degree of immunity followed infection with vaccinia virus by either route and that the immunity was recognizable by all the routes used for challenge. The dilution of virus suspension used in the case of strain "B" was greater than that used for "A", which may account for the larger number of survivors.

Discussion. We are told that ectromelia is frequently present in European mouse colonies and has at times greatly complicated mouse experiments. The danger of introducing it into domestic stocks is evidently very real and infected mouse tissues should be held suspect until the presence of ectromelia is excluded.* Probably an early inoculation into the footpad would be a simple and effective precaution. Whether the unusual behavior of our animals was due solely to the method of inoculation or the fact that our stock is not latently infected or both we cannot say. It is interesting to note that a number of European workers have reported that latent infections of their stock mice have confused their studies of influenza(8). Intranasal inoculation of such mice with human nose and throat washings has apparently induced pneumonia due to ectromelia rather than influenza virus. Shortly before we recognized the nature of these two strains of virus we had inoculated litters of suckling mice intranasally. The larger doses caused illness and death within 3 days and areas of consolidation were found in the lungs of some mice. At this point these mice were also destroyed.

The experience confirms the value of thorough post-mortem examination of experimental animals and the usefulness of routine histologic examinations of unidentified gross lesions. Anatomic observations are relatively simple and often most rewarding. They should not be neglected.

Summary. Two viruses believed to be neurotropic human infections have been identified as mouse ectromelia. The behavior of ectromelia in young mice inoculated intracerebrally is described.

* A committee of the Virus and Rickettsial Study Section of the Division of Research Grants, National Institutes of Health, has recently published a warning of the danger of disseminating infectious ectromelia and the basic facts concerning it.(7)

1. Marchal, J., *J. Path. and Bact.,* 1930, v33, 713.
2. Melnick, J. L., and Gaylord, W. H., Jr., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 315.
3. Trentin, J. J., *Science,* 1953, v117, 226.
4. Schoen, R., *Comp. Rend. Soc. de Biol.,* 1938, v128, 695.
5. Fenner, F., *J. Immunol.,* 1949, v63, 341.
6. Cheever, F. S., Daniels, J. B., Pappenheimer, A. M., and Bailey, O. T., *J. Exp. Med.,* 1949, v90, 181.
7. Shope, R. E., *J. Lab. and Clin. Med.,* 1954, v44, 333.
8. Kikuth, W., and Gönnert, R., *Arch. Virusforsch.,* 1940, v1, 295.

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Pharmacological Properties of Recanescine, A New Sedative Alkaloid from *Rauwolfia canescens* Linn. (21567)

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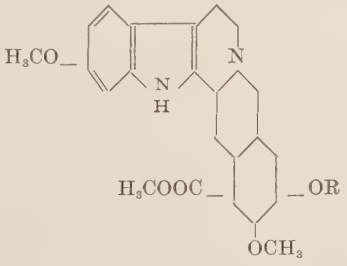
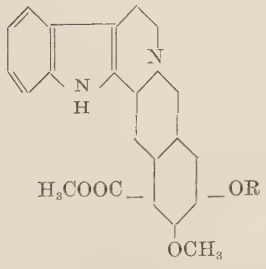
Rauwolfia canescens Linn, a species of the family of Apocyanaceae, is closely related to *Rauwolfia serpentina* Benth. On occasion it has been found as an adulterant in the commercial lots of the latter. The alkaloidal content of the plant has been investigated by several workers within the last year(1-3) leading to isolation of rauwolscine, reserpine, serpentine and yohimbine.

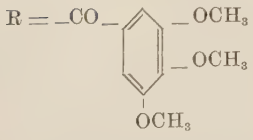
We have been able to isolate and characterize a new sedative alkaloid which we called recanescine (Lilly No. 22641). It is a weak base closely related to reserpine. All the physical and analytical data indicate that recanescine is 11-desmethoxyreserpine.*



Reserpine



Recanescine



* Physical and chemical properties of recanescine will be described in detail elsewhere.

On the basis of evidence from paper chromatography, infra-red and ultraviolet spectroscopy, our sample of recanescine appears to be homogenous material free from other alkaloids of the plant. The pharmacological data indicate that recanescine has properties similar to, or identical with, those of reserpine. This finding should simplify considerably the chemical synthesis of effective drugs of this type.

Methods and materials. The recanescine used in this study was available as an ethyl acetate solvate. It is readily soluble in glacial acetic acid and when diluted makes a slightly yellow solution of pH 3-4. This solution, in suitable concentration, was injected intravenously into mice, rats, rabbits, cats and monkeys. In the monkeys and rabbits only the degree of sedation, ptosis and miosis was recorded. In rats, the amount of current necessary to produce a maximal tonic extensor seizure was determined using corneal electrodes and the electro-stimulator(4). The effect of reserpine and recanescine on Evipal sleeping time of mice given 1 mg/kg subcutaneously was determined(5). Seven cats were lightly anesthetized with ether and then given alpha-chloralose (40-80 mg/kg).† Arterial blood pressure was recorded with a mercury manometer. The effect of occlusion of the carotid arteries and of stimulation of the tibial nerve centrally, the superior cervical nerve peripherally and the vagus both centrally and peripherally has been observed. Epinephrine (5 μg/kg), methacholine (1 μg/kg) and histamine (5 μg/kg) were injected both before and

† Alpha-chloralose is readily soluble in polyethylene glycol 200 [carbowax]. A 10% solution can be prepared by adding the drug to hot solvent. The solution remains clear at room temperature. This procedure makes the routine use of alpha-chloralose for anesthesia a convenient practical method quite in contrast with the older practice of injecting the drug as a 1-1.5% solution in hot water.

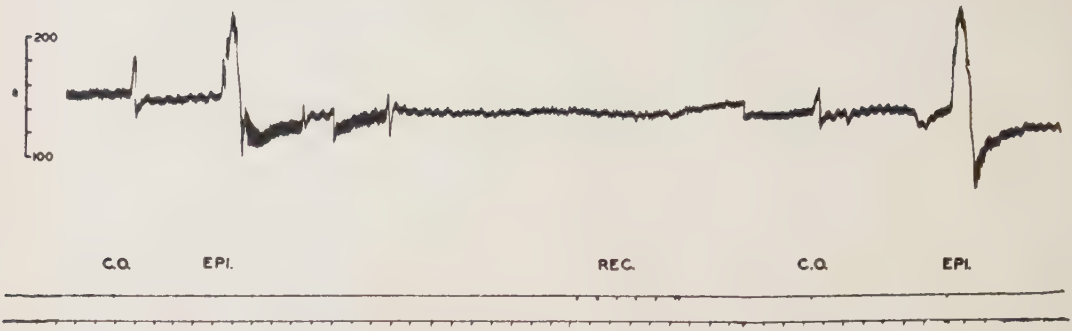


FIG. 1. Effect of recanescine, 1 mg/kg, on response of arterial pressure to carotid occlusion (C.O.) and epinephrine (EPI), 5 μg/kg. Cat—5.25 kg. Chloralose anesthesia, 60 mg/kg. Time, 1 min. Break in record indicates one hour.

after the injection of recanescine. Whenever possible, similar experiments have been done with reserpine in the same dosage.

Results. When recanescine (1 mg/kg) was injected slowly in cats anesthetized with alpha-chloralose, a transient fall, or rise, or no change in mean arterial pressure occurred. After 60-90 minutes, however, a slow consistent decrease in blood pressure and pulse rate became evident. This fall continued through the second and third hour during which time the electrocardiogram remained unchanged except, of course, for the slowing of rate. During the hypotension following 1 mg/kg of recanescine, the pressor and cardioaccelerator effects of bilateral carotid artery occlusion usually diminished. This phenomenon was noted only after the blood pressure had fallen 25-40 mm Hg. Similarly, the pressor effect of central vagus stimulation, when present prior to recanescine, was diminished after the drug. In 2 experiments, a depressor response replaced the pre-drug pressor response.

The pressor response to epinephrine usually was enhanced during recanescine induced hypotension. In each case the absolute increase in mean arterial pressure was greater. In half the experiments, the blood pressure after epinephrine rose to a higher peak despite the lower basal pressure. The response of the nictitating membrane to the injection and to preganglionic stimulation was not changed. This finding suggests that the increased responsiveness is a consequence of the low blood pressure and obtunded vasomotor reflexes rather than an inherent increased responsiveness to epinephrine. The depressor effects of methacholine and histamine were not modified by recanescine.

The pressor response which was noted during repetitive stimulation of the tibial nerve at 10-100 c/sec was decreased after the drug. During the stimulation, low voltage fast activity replaced for 10-15 seconds the slow activity and spikes typical of the electroencephalogram under light chloralose anesthesia. The injection of recanescine (1 mg/kg) did not appear to modify this arousal pattern. The flexion reflex elicited by stimulation of the tibial nerve with brief single pulses at 10 second intervals was not decreased and, indeed, in some experiments was enhanced after the injection of recanescine.

Our findings in these acute experiments are consistent with the view that recanescine, like reserpine, exerts its depressor effect, at least in part, through a change in the reactivity of central vasomotor control.

The effect of recanescine and reserpine was compared in monkeys. In one experiment a nursing female was given 1 mg/kg of recanescine. The animal showed the often described sedated appearance of a monkey given reserpine(6). Her aggressive resentment of our handling the baby monkey virtually disappeared for several hours. This monkey and 6 others given 0.5 or 1.0 mg/kg of recanescine developed miosis and ptosis. One monkey showed ptosis, sedation, but no pupillary constriction.

In another experiment doses of 0.5 or 1.0 mg/kg of recanescine and reserpine were given to 12 monkeys in groups of 3, selected in a random fashion. The observer, who had

worked with these animals before, did not know the distribution of the various doses. The degree of sedation was graded on a scale from 1 to 3. None of the animals showed the deep sedation for which the 4+ category had been reserved. Table I summarizes the results of this experiment. In tabulating these data the sedation score for each group of animals was added and recorded as a fraction of the total of 9 pluses possible for a group of 3 monkeys graded on a 3 point scale. It would appear that after the second hour it was possible to distinguish between the sedative effects of 0.5 and 1.0 mg/kg of the alkaloids but not between the alkaloids.

Assuming the drugs to be equally effective and applying a simple "t" test to the difference in mean responses to the 2 dose levels, a figure of 2.89 is obtained which would indicate that for odds of 98-1 it is possible to discriminate between the 2 doses used. After adjusting the responses on the low dose for the dosage response relationship, the "t" test may be applied to the difference between mean responses to the two drugs. The figure obtained, 0.18, would indicate that a difference as great could occur 80 times in 100 simply due to random chance, so no discrimination is possible between drugs with these data. When recanescine was given to mice or rabbits, a typical reserpine-like depression with ptosis was noted. Here again, the two alkaloids appeared approximately equipotent. Reserpine causes a decrease in convulsant threshold(7). In Table II there is recorded an experiment in which the current necessary to produce an extensor convulsion in 100 g rats was determined. Here again, a similarity of reserpine and recanescine is demonstrated. 1 mg/kg of either reserpine or recanescine significantly prolongs Evipal sleeping time.

TABLE I. Comparison of Sedative Properties of Reserpine and Recanescine in Rhesus Monkeys.*

	Dose	n	Degree of sedation 1 hr	2 hr	3 hr	4 hr
Reserpine	.5	2†	1/6	2/6	3/6	2/6
	1.0	3	4/9	7/9	7/9	7/9
Recanescine	.5	3	4/9	4/9	5/9	5/9
	1.0	3	3/9	7/9	7/9	6/9

* Sedation graded 1-3 making a possible 9 in each group.

† Third monkey in this group died of strangulation when his chain caught in the floor meshing.

TABLE II. Comparative Effect of Reserpine and Recanescine on Convulsive Dose 50% in Rats by Maximal Electroshock Seizure Method. Thirty animals in each series.

Treatment	Dose, mg/kg	Route	$CD_{50} \pm$ S.E. in ma
Untreated	—	—	35.55 ± 2.56
Reserpine	.5	I.P.	24.64 ± 1.03
Untreated	—	—	34.97 ± 4.37
Recanescine	.5	I.P.	21.53 ± 1.06

By this method the 2 drugs cannot be distinguished.

Summary. A new alkaloid isolated from *Rauwolfia canescens,* recanescine, appears to lack the methoxyl group in the C-11 position of reserpine. Preliminary evidence indicates that this compound retains the characteristic pharmacologic activity of reserpine. Thus, the methoxyl group is not essential for the sedative and hypotensive action of reserpine.

Note added in proof. Since submission of this manuscript, Stoll and Hofmann have reported the isolation of a new alkaloid, canescine, which is probably identical with our recanescine. (Stoll, A., and Hofmann, A., *J. Am. Chem. Soc.,* 1955, v77, 820.)

1. Chatterjee, A., *Naturw.,* 1954, v41, 215.

2. Klohs, M. W., Draper, M. D., Keller, F., and Petracek, F. J., *J. Am. Chem. Soc.,* 1954, v76, 1381.

3. Haack, E., Popelak, A., Springler, H., and Kaiser, F., *Naturw.,* 1954, v41, 479.

4. Toman, J. E. P., Swinyard, E. A., and Goodman, L. S., *J. Neurophysiol.,* 1946, v9, 231.

5. Cook, L., Toner, J. J., and Fellows, E. J., *J. Pharmacol. and Exp. Therap.,* 1954, v111, 131.

6. Plummer, A. J., Earl, A., Schneider, J. A., Trapold, J., and Barrett, W., *Ann. N. Y. Acad. Sci.,* 1954, v59, 8.

7. Jenney, E. H., *Fed. Proc.,* 1954, v13, 370.

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Histochemical Demonstration of Glycogen in the Mouse Pituitary. (21568)

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Cytological studies of the anterior pituitary gland are often based on the use of the periodic acid-Schiff (PAS) technic because of the positive reaction of mucoproteins which are localized exclusively in the "basophil" cells, and which are believed to represent the several mucoprotein hormones or their precursors (1,2). Other substances in fixed animal tissues, besides mucoprotein, which react with the PAS method, are glycogen, mucopolysaccharides, glycoproteins and glycolipids.

It became apparent during the course of a study of the pituitaries of normal mice, that cells of the pars tuberalis, and certain other structures in the pituitaries of very young animals, gave an even more pronounced reaction with PAS than is seen in the basophils. As the pars tuberalis cells are typically described as being largely chromophobic, and as the staining of the neonatal pituitaries was of an apparently undescribed character, it seemed of interest to investigate the nature of the reactive material. The application of saliva or malt diastase to pituitary sections prior to staining with PAS prevented the reaction in the specific sites described above, thus providing histochemical evidence that glycogen was responsible for this unusual staining pattern.

Methods. Thirty-four normal mice of the first generation of the cross BALB/c X C_3H were used. They were of both sexes and were from 1 day to 7 months old. Pituitaries were fixed in the skull after removing the parietal bones and pulling back the brain or, in some cases, trimming the brain to a small piece just above the pituitary so as to preserve intact the stalk, pars tuberalis and median eminence of the hypothalamus. Tissues were fixed 18 to 24 hours in buffered sublimate-formol having a pH of about 5.8(3). After fixation, tissues were washed briefly in tap water, pituitaries were dissected from the skulls, in some cases together with stalk and hypothalamus, dehydrated, cleared and embedded. Paraffin sections were cut serially. The periodic acid-Schiff method was employed using aqueous reagents as outlined by Lillie(4). Suitable sections for testing with saliva or diastase digestion were selected from 10 of the 34 pituitaries. In testing for glycogen, sections were exposed at room temperature to saliva for 20 to 30 minutes, or to 1% Malt Diastase U.S.P. Fisher IX in buffer solution (5), prior to the PAS procedure. When possible two adjacent sections, one of which had been treated with the enzyme, were stained simultaneously. In every case, slides of pituitaries known to contain the strongly PAS-reacting substance in question were stained without previous digestion in order to test the staining procedure.

Results. Use of the PAS stain on the pituitaries of young mice showed the basophils of the anterior lobe and the cells of the intermediate lobe to be stained the pink-to-red color usually described for these sites. In addition, however, this technic revealed a brilliant magenta-staining material of a somewhat amorphous appearance in most of the cells in the pars tuberalis of the young animals. (Fig. 1, 3, 5, 6, 7) This material was abundant throughout the first month of life after which time only occasional magenta granules in diffuse purplish cytoplasm are seen. This latter form of staining could still be observed at age 7 months (Fig. 8).

In addition to the pars tuberalis, highly PAS-positive material was seen in mice one to five days of age in the sinusoids of the anterior lobe, in the cells and the intercellular spaces of the neural lobe, and in the stalk and the adjoining median eminence. Between 5 and 8 days the heavy staining in the anterior lobe disappeared and was diminished in the neural portion. By 11 days it was seen

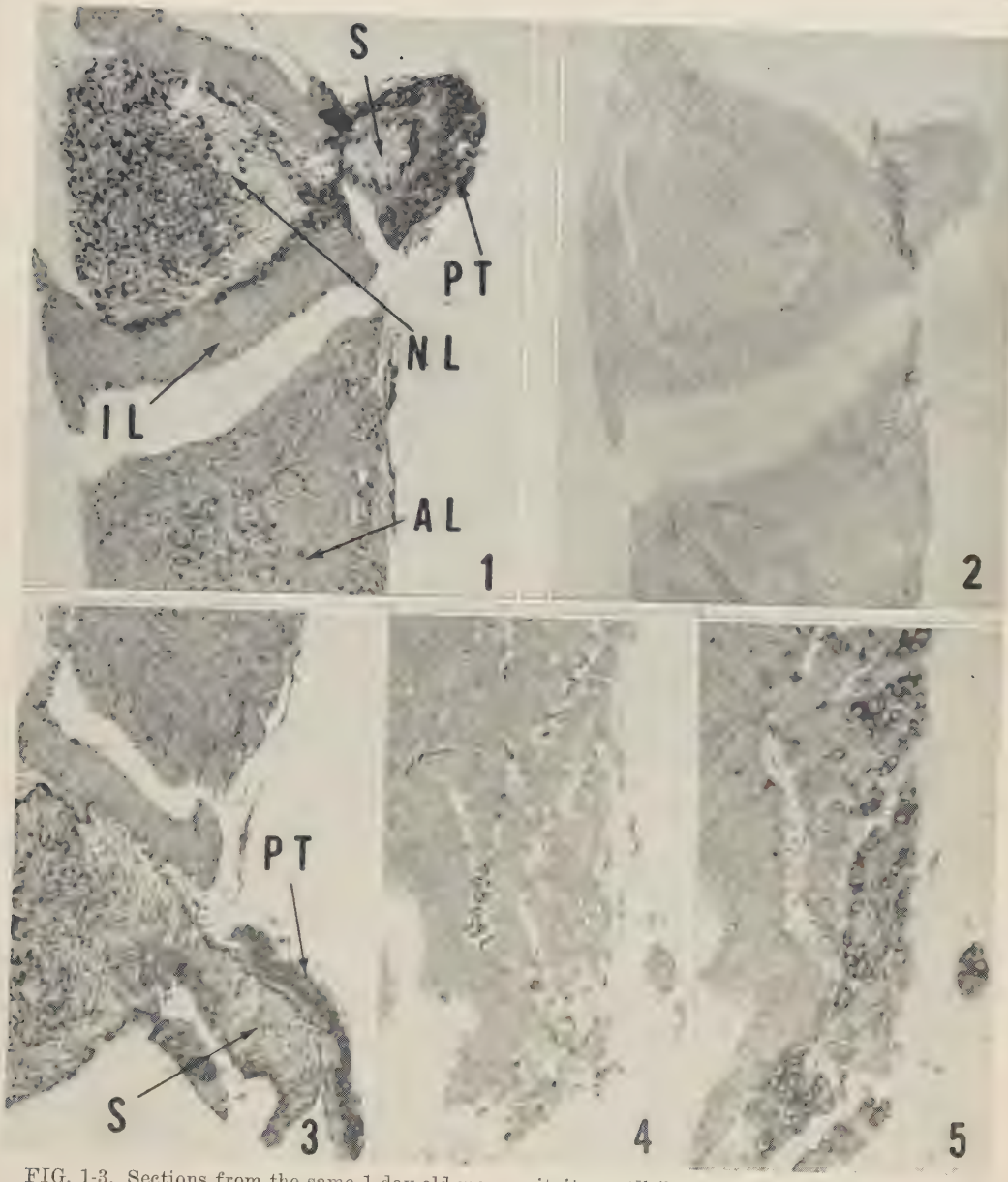

FIG. 1-3. Sections from the same 1-day-old mouse pituitary, all 5 μ thick, $\times$ 130. AL, anterior lobe; IL, intermediate lobe; NL, neural lobe; PT, pars tuberalis; S, stalk.

FIG. 1. PAS, hematoxylin and orange G. The magenta-staining material appears black, the faint pink staining appears as light grey.

FIG. 2. Saliva, PAS. Serial sections ventral to Fig. 1, illustrating total loss of magenta staining material following saliva digestion, while the faint PAS reaction can be discerned.

FIG. 3. Stained as Fig. 1, section from more dorsal part of gland.

FIG. 4 and 5. Adjacent sections from a 5-day-old male mouse pituitary, showing pars tuberalis cells at rostral border of anterior lobe, $\times$ 260.

FIG. 4. Masson's stain (a modified Mallory Triple). Pars tuberalis cells appear empty as they are unstained or very faintly blue. Compare with Fig. 5.

FIG. 5. PAS, hematoxylin and orange G. By contrast with Fig. 4, pars tuberalis is deeply stained by PAS.

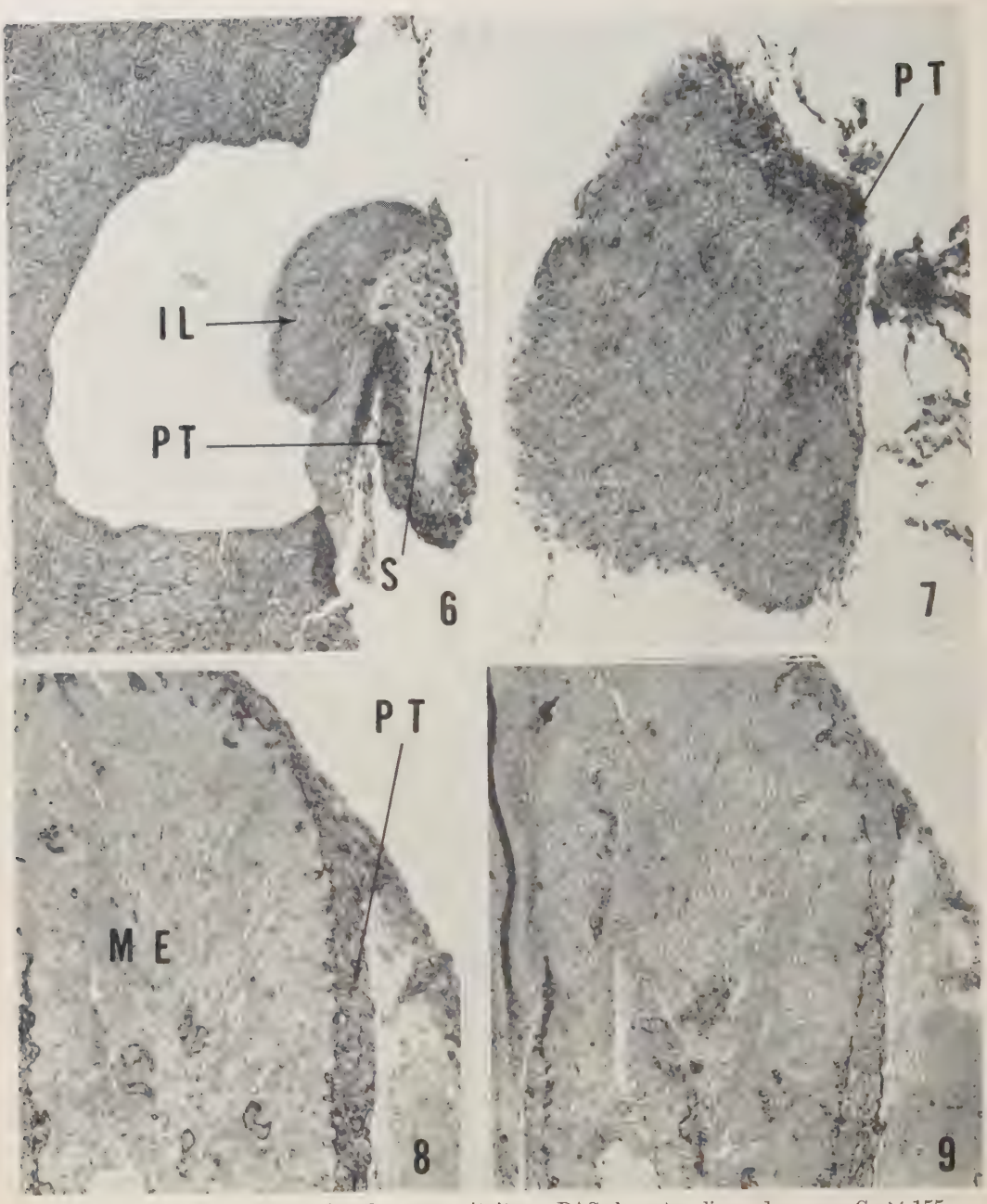

FIG. 6 and 7. 3-day-old female mouse pituitary, PAS, hematoxylin and orange G, × 155. Fig. 7, considerably ventral to Fig. 6, shows pars tuberalis as dark-staining V-shaped area embedded in anterior lobe tissue.

FIG. 8 and 9. 7-months-old female mouse, adjacent sections of pars tuberalis in region of the median eminence of hypothalamus, × 155.

FIG. 8. PAS, hem., orange G. Nervous tissue stained pink. Pars tuberalis is stained diffusely purple.

FIG. 9. Diastase, PAS, hem., and orange G. Nervous tissue also pink as in Fig. 8, but pars tuberalis cells are unstained.

only in the pars tuberalis. It may be mentioned that this type of staining was not seen in the intermediate lobe except for some globules on either surface adjacent the anterior or neural lobes in the youngest animals (as in Fig. 1 and 3). The magenta staining in the anterior lobe had an amorphous, homogeneous appearance, while that in the neural portions of the pituitary complex was heavily granular or globular.

Diastase or saliva treatment completely prevented the strongly positive magenta staining in all of these sites. On the other hand, the enzymatic digestion did not remove the finely-granular *pink to red* staining of the basophils, the intermediate lobe cells, or of the intercellular areas of the neural lobe. In view of the fact that glycogen is the only PAS-positive substance in fixed animal tissues known to be removed by saliva or diastase(6) it seems reasonable to conclude that the heavily staining material described above is glycogen. It should be noted that the presumed glycogen was not stained by Schiff's reagent without previous periodate oxidation, thus indicating an actual PAS reaction.

The presence of a stainable material in the cells of the pars tuberalis made it easy to trace the extent of this portion of the pituitary which is otherwise remarkably inconspicuous. In sections stained by a modified Mallory triple method, the pars tuberalis, identified with the help of the adjacent PAS-stained sections is either slightly stained by aniline blue or, more often, chromophobic (Fig. 4 and 5). The nuclei of the tuberal lobe are smaller than those of the anterior lobe, and the cells themselves are generally smaller than acidophils. The glycogen-containing pars tuberalis was seen to extend from the rostral and ventral point of the pituitary stalk where it emerges from the median eminence of the hypothalamus, caudally along the stalk to an abrupt end dorsally where the intermediate lobe encloses the stalk as it enters the neural lobe. On the ventral surface the pars tuberalis is still in contact with the stalk as it meets the rostral border of the anterior lobe. Here the pars tuberalis cells extend somewhat laterally and actually penetrate within the anterior lobe proper, where they can be seen between some of the large sinusoids of the portal system (Fig. 7).

The unexpected appearance of glycogen in these mouse pituitaries raised the question of whether the buffered fixative allowed the visualization of glycogen not ordinarily preserved by the more commonly employed unbuffered sublimate-formol (pH 3). However, examination of newborn mouse pituitaries fixed in unbuffered sublimate-formol ruled out this possibility as there was no detectable difference in staining between these pituitaries and those fixed at pH 5.8.

Discussion. The significance of the presence of glycogen in the pars tuberalis, and in the anterior and neural lobes of neonatal mice, cannot at present be stated. Although it is tempting to point to the sugar content of 3 of the pituitary hormones as possibly indicating a need for carbohydrate stores in this organ, there is no information which would permit the inference of a specific relationship between these two phenomena. Furthermore, the appearance and subsequent depletion of a considerable amount of glycogen in the newborn pituitary in the first week of life has a parallel in an experiment by Hinde on the neonatal kidney(7). In the experiment referred to, abundant deposits of glycogen were demonstrated with the PAS technic as intensely staining magenta granules in the cells of the collecting tubules of the newborn rabbit. In contrast, only traces of glycogen were found in this site after the third week of life. Examination of kidneys of the newborn and adult guinea pig, rat and man gave the same results, though the amount of glycogen in the cells of the neonatal collecting tubules was less in these species than in the rabbit. The author raised the question of whether the glycogen in the neonatal kidney was mobile or had a local function, that is, whether like liver or like muscle glycogen. The significance of this question is underlined by the data which indicate depletion of carbohydrate stores shortly after birth when the fall in the respiratory quotient reveals a shift from utilization of carbohydrate exclusively to utilization of fat(8). Indeed the liver glycogen of the guinea pig was shown to drop from greater than adult levels at birth to almost nothing in

10 hours(9). It would be interesting to know whether the glycogen seen in neonatal pituitaries and kidneys represents accessory depots of carbohydrate for the general body metabolism of the newborn, or whether it has a specific role in the development of the organ or in its early postnatal function. The presence of histologically demonstrable amounts of glycogen in the pars tuberalis at 7 months might suggest a special significance of glycogen to the pituitary function of the adult.

A noteworthy feature of the glycogen in young pituitaries is its distribution in the most vascular areas of the pituitary complex; it was not localized in the relatively avascular intermediate lobe or in areas of the brain adjacent the very vascular median eminence. This preference for localizing in the more vascular parts of the organ was not the case with the newborn kidney.

Summary. 1. Material giving a very strong periodic acid-Schiff reaction was seen in certain structures in sections of mouse pituitaries. This material was shown to be glycogen by its disappearance when exposed to saliva or diastase prior to staining. 2. In mice 8 days and younger, glycogen could be demonstrated in the intercellular spaces and in the cells of the neural lobe, in the cells of the pars tuberalis, and intercellularly in the stalk and median eminence of the hypothalamus. At 5 days and less, glycogen also appeared to be located in irregular patterns in the sinusoids of the anterior lobe. 3. After 8 days of age, glycogen was apparently restricted, in the pituitary system, to the pars tuberalis. After one month the amount of glycogen was considerably reduced, but nevertheless could still be demonstrated at 7 months.

Appreciation is expressed to Dr. Monte A. Greer for valuable advice and criticism, to Dr. Clifford Grobstein for providing the animals, and to Mr. John McGuire for taking the photographs.

1. Catchpole, H. R., *J. Endocrinology,* 1949, v6, 218.
2. Pearse, A. G. E., *J. Path. Bact.,* 1949, v61, 195.
3. Lillie, R. D., *Histopathologic Technic and Practical Histochemistry,* 1954, New York.
4. ———, *J. Lab. Clin. Med.,* 1947, v32, 910.
5. ———, *Stain Tech.,* 1947, v22, 67.
6. Pearse, A. G. E., *Histochemistry,* 1953, London.
7. Hinde, Isobel T., *J. Path. Bact.,* 1949, v61, 451.
8. Smith, C. A., *The Physiology of the Newborn Infant,* 1951, Springfield, Ill.
9. Hard, W. L., Reynolds, O. E., and Winbury, M., *J. Exp. Zool.,* 1944, v96, 189.

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A New Micro-Method for the L.E. Cell Phenomenon.* (21569)

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The primary role of the polymorphonuclear blood cells in the L.E. cell reaction has been demonstrated in supravital studies by Rohn and Bond(1). In the test being described, a film of polymorphonucleocytes has been placed in contact with the lupus erythematosus (L.E.) factor of the patient's serum on a glass surface. This environment allows them free movement and does not interfere with phagocytic ability. The method shows promise not only as a diagnostic aid, but also as a useful method for investigative work.

Method. Living granulocytes are isolated on a glass slide by a modification of the method described recently by Harris(2) and originally by Wright and Colebrook(3). The cells are then exposed to the L.E. factor in serum or plasma. The following are the steps. Granulocytes from any donor are obtained by depositing 2 to 3 drops of blood obtained intravenously or by finger puncture in each of 3 circles on a clean glass slide. The slide is then incubated in a closed Petri dish containing moistened filter paper at 37°C for 20 min-

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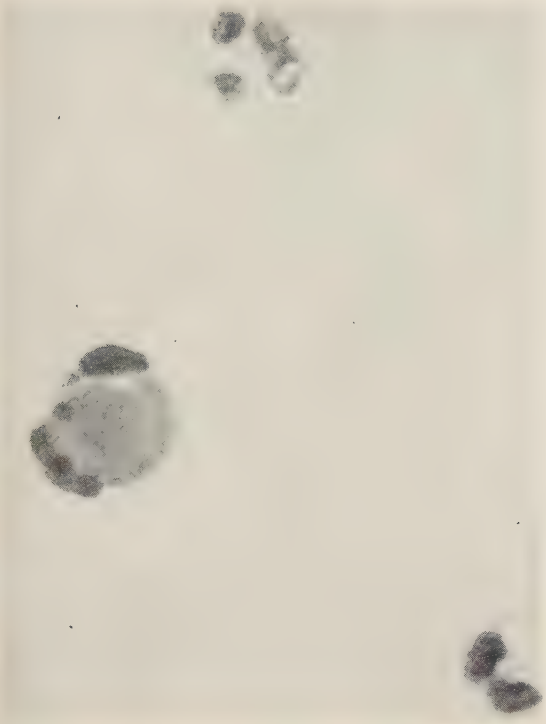

FIG. 1. L.E. cell surrounded by polymorphonucleocytes of "disc."

utes. It is then thoroughly washed by a spray of normal saline from a wash bottle to remove the clot and wash away all extraneous elements. A thin, filmy "disc" remains in each circle which consists almost entirely of polymorphonuclear leucocytes. The "discs" are then flooded immediately with several drops of the plasma or serum to be tested, and promptly covered with cover slips. The slides are then again incubated at 37°C in closed Petri dishes containing moistened filter paper for one hour. The cover slip is removed; the slide is allowed to air dry completely while standing on edge. It is then stained routinely with Wright or MacNeal tetrachrome, and examined under the microscope. Six discs should be examined before reporting a negative result. If the polymorphonucleocytes have been properly preserved they will show normal structure on staining. If they appear abnormal, it is likely that they have lost their phagocytic activity and that the test will not be successful.

Results. The "disc" test gave characteristic L.E. cells with the serum of 11 patients with systemic lupus erythematosus(Fig. 1). It was negative in one patient with chronic disseminated lupus erythematosus whose serum had reacted negatively repeatedly by a number of standard procedures including the two-hour clot method(4). Of the 11 positive sera, 8 were from patients with the acute form of the disease, who at the time were being maintained in almost complete remission on suppressive therapy. L.E. cells were noted in 36 different sera of 41 tested in this group. The sera of 2 patients with subacute disseminated lupus erythematosus gave positive tests. One patient with low grade chronic disseminated disease gave a positive test on only one of 8 separate serum samples.

Control sera were obtained from 26 normal individuals, 36 patients with active rheumatoid arthritis, 33 patients with active rheumatic fever, and 24 with a number of miscellaneous conditions. All gave negative results.

In all 29 determinations on individual sera from 10 patients, in which the present modification and the 2-hour clot method(4) were carried out simultaneously, the "disc" test showed either as many or more L.E. cells per high power field than the latter test. The "disc" method, moreover, was often positive when the clot method yielded results which were either negative or difficult to interpret with certainty.

In addition to formation of characteristic L.E. cells, rosette formation and clumping of leucocytes were frequently observed. Rosette formation and clumping without true L.E. cell formation were uncommon.

Discussion. Positive results by the method described are easily distinguished. With the elimination of almost all red blood cells, platelets, and mononuclear elements the likelihood of false positive reading is reduced. The granulocytes are not distorted since they are not subjected to mechanical trauma. The ease with which a negative result can be established is also an important advantage of the "disc" method. A rapid scanning under low power is usually sufficient to obtain an accurate reading.

Clumping of granulocytes was frequently observed when sera from patients with lupus

erythematosus were tested. By the method described, and in the granulocyte studies of Harris(2), normal polymorphonuclear cells do not clump and are found to lie distinctly separated from one another on the slide. Clumping of cells, only, without true rosette or L.E. cell formation has been observed by us as an isolated finding, moreover, only in patients who have at other times demonstrated true L.E. cells. It would appear likely, therefore, that the occurrence of clumping using the "disc" test is an indication of the presence of L.E. activity either as part of the L.E. cell phenomenon or due to a separate "clumping factor."

Summary. A micro-method for the demonstration of the L.E. cell in peripheral blood employing a "disc" of granulocytes spread on glass has been presented. It has the advantage of ease of performance, simplicity in reading, and increased sensitivity.

We wish to thank Mr. M. Tanner for technical assistance, and Mr. B. Joffe for aid in these studies.

1. Rohn, R. J., and Bond, W. H., *Am. J. Med.*, 1952, v12, 422.

2. Harris, H., *J. Path. and Bact.*, 1953, v66, 135.

3. Wright, A. E., and Colebrook, L., London, 1921, 268.

4. Zimmer, F. E., and Hargraves, M. M., *Proc. Staff Meet. Mayo Clin.*, 1952, v27, 22, 414.

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Role of Duodenum in the Control of Gastric Secretion. (21570)

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That the duodenum plays an important role in the control of the secretory and motor activity of the stomach was demonstrated long ago, when Sokolov(1) in Pavlov's laboratory demonstrated that the inhibitory effect which fat was known to have on secretion of gastric juice was mediated through the duodenum rather than by some direct effect of fat upon the stomach itself. At the same time, Sokolov reported that the introduction of 0.5% HCl of gastric juice into the duodenum inhibited gastric secretion. These observations were made on Pavlov-pouch dogs with gastric and duodenal fistulas. Sokolov's findings indicated that both nervous and chemical phases of gastric secretion were inhibited.

The experiments reported herein were devised to try to obtain some indication as to the nature, magnitude and possible importance of the inhibitory control of the duodenum over gastric secretion. That such control may be of importance is suggested in experiments of Storer, Oberhelman, Woodward, Smith and Dragstedt(2) who showed that there was an increase in the amount of gastric juice secreted by an isolated stomach pouch following the Exalto-Mann-Williamson procedure in which the segment of the duodenum containing the terminations of the pancreatic and bile ducts are drained into the terminal ileum. Some suggestion of duodenal control over gastric secretion is indicated also in experiments of Keefer, Martin and Glenn(3), who found that direct transplantation of the duodenum into the stream of the terminal ileum or of the colon, resulted in development of perforated duodenal ulcers in a high percentage of dogs.

Methods. This report is based on data obtained from 8 adult male and female mongrel dogs weighing from 10 to 20 kg. Each dog had 2 operations. First, an isolated gastric (Heidenhain) pouch was fashioned from the greater curvature of the body and fundus of stomach—this pouch was drained externally by means of a lucite cannula protected externally by means of a stainless steel sleeve. The dogs were allowed to recover from this first operation for approximately 3 weeks, and then the output of their Heidenhain pouches was "standardized." In standardizing, the pouch secretion was collected for an 8 hour period, every other day, 3 days a week. Dogs were fasted 16 hours prior to each collection

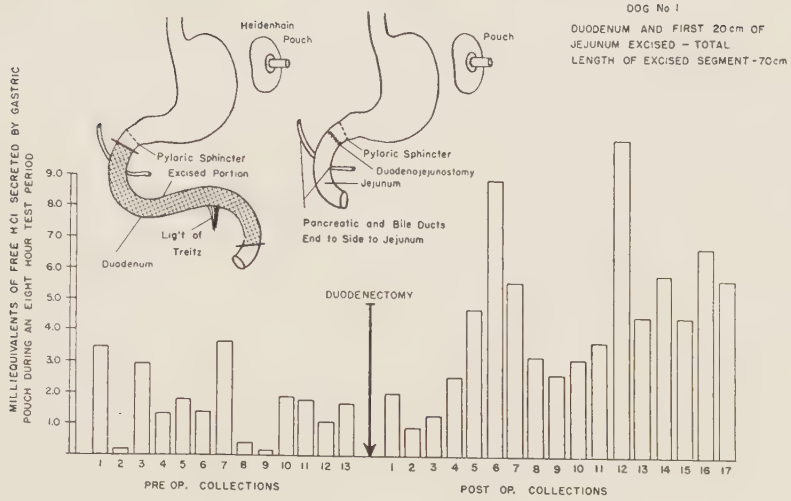

FIG. 1. Effect of resection of the duodenum and proximal jejunum on secretion of HCl by a Heidenhain pouch.

period so that gastric secretion would be in a basal, fasting state at beginning of collection, and then at the end of second hour of collection period they were given a meal of 200 g of cooked horse meat as a standard stimulus to gastric secretion. The volume of secretion and the degree of free acidity and of total acidity were measured. The amount of free HCl in milliequivalents secreted during collection period was then calculated by multiplying the free acidity in clinical units by volume of secretion in liters. These standardizing collections were made 3 days a week for at least 4 weeks. After pouches were standardized, the dogs were divided into 2 groups and were operated upon a second time. In *Group* 1 (Duodenectomy) the duodenum, except for the first 2 cm distal to the pylorus, and the proximal 20 to 30 cm of jejunum were excised. During the dissection, care was taken to preserve the blood supply of the pancreas. Then the jejunum was anastomosed end-to-end to the duodenal remnant on the pylorus, and the common bile duct and the pancreatic duct were anastomosed end-to-side to the jejunum in approximately the same relation to the pylorus that their original outlets into the duodenum had. The 2 cm remnant of duodenum was left attached to the pylorus to preserve, if possible, normal pyloric sphincter function. (See Fig. 1 for a diagram of this operation.) In *Group* 2 (Duodenal Transplant), the entire duodenum, except for the first 2 cm distal to pylorus, and the proximal 20 to 30 cm of the jejunum were transplanted into the small bowel at about mid-point of the jejuno-ileum. In other words, a segment of jejuno-ileum approximately 1.5 meters long was interposed between stomach and duodenum. This was done by transecting the duodenum about 2 cm distal to the pylorus and dividing the jejunum about 30 cm distal to ligament of Treitz. Bile and pancreatic ducts remained intact in the duodenojejunal segment transplanted to its new lower level in the intestinal tract. The distal cut end of the jejunum was then anastomosed end-to-end to the remnant of duodenum left attached to the pylorus. The jejuno-ileum was then transected at approximately its mid-point, and the isolated duodenojejunal segment was transplanted isoperistaltically into this point by making end-to-end anastomoses between proximal end of the isolated segment and proximal cut end of the jejunoileum and between the distal end of the isolated segment and the distal cut end of the small bowel. (See Fig. 2 for a diagram of this operation.) The dogs were allowed to recover from their second operation for approximately one week, and then the output of free HCl from their isolated gastric (Heid-

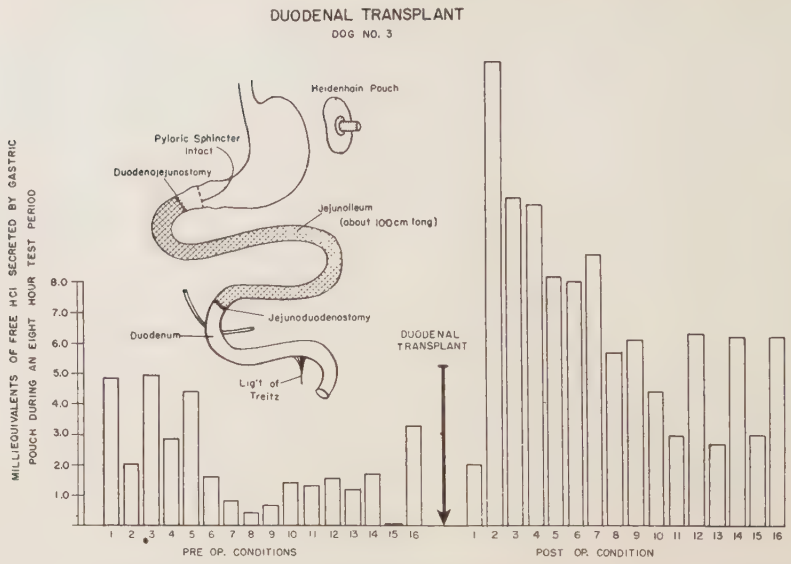

FIG. 2. Effect of interposition of a long segment of jejunoileum between the stomach and duodenum on the secretion of HCl by a Heidenhain pouch.

enhain) pouches was again standardized. This standardization was carried out in exactly the same way as the original pouch standardization, except that collections were continued every other day, three days a week for as long as the animals lived. Generally speaking, neither duodenectomy nor duodenal transplant operation seemed to have any adverse effect on the dogs' general condition. All dogs had good appetites postoperatively, and they maintained their weights well. A few of them died of distemper and of intussusception. Autopsy of these dogs revealed that all anastomoses, both of bowel and of pancreatic and bile ducts, were widely patent with no suggestion of narrowing or obstruction.

Results. A marked increase in the amount of free HCl secreted during the 8 hour collection periods from the Heidenhain pouches of all dogs, followed both duodenectomy and duodenal transplantation; and the magnitude of the increase was roughly the same following both operations.

Table I summarizes the data for Group 1 (Duodenectomy). Following resection of duodenum and proximal jejunum, the increase in mean secretion of free HCl per 8 hour collection period ranged from 84% in one animal to 820% in another. The difference between pre-operative and post-operative mean secretion levels is statistically significant in every case. Generally speaking, dogs with the smaller preoperative outputs had the greater

TABLE I. Mean Output of Free HCl by Isolated Gastric (Heidenhain) Pouches before and after Resection of Duodenum and Proximal 20 to 30 cm of Jejunum.
Group 1, duodenectomy in 5 dogs.

Pre-operative			Post-operative					
No. of samples	Mean output of HCl/8 hr in mEq ($\bar{X}_1$)	Stand. dev.	No. of samples	Mean output of HCl/8 hr in mEq ($\bar{X}_2$)	Stand. dev.	Diff. between means ($\bar{X}_1-\bar{X}_2$)	Stand. error of diff.	% increase following operation
13	1.536	1.340	19	4.508	2.471	2.972	.675	194
15	1.595	1.500	30	4.274	3.552	2.679	.756	168
13	.288	.452	68	2.649	1.635	2.361	.233	820
13	5.788	1.875	6	10.63	3.821	4.842	1.470	84
12	4.012	1.648	6	11.32	4.209	7.308	1.783	182

TABLE II. Mean Output of Free HCl by Isolated Gastric (Heidenhain) Pouches before and after Interposition of a Segment of Jejuno-Ileum Approximately 1.5 Meters Long between the Stomach and Duodenum.

Group 2, duodenal transplant, 3 dogs.

Pre-operative			Post-duodenal transplant					
No. of samples	Mean output of HCl/8 hr in mEq ($\bar{X}_1$)	Stand. dev.	No. of samples	Mean output of HCl/8 hr in mEq ($\bar{X}_2$)	Stand. dev.	Diff. between means ($\bar{X}_1-\bar{X}_2$)	Stand. error of diff.	% increase following operation
14	3.101	1.810	32	6.580	3.883	3.479	.840	113
13	.558	.669	53	2.590	1.831	2.032	.321	364
15	2.023	1.765	29	5.148	3.384	3.125	.776	154

percentage increase in pouch output post-operatively.

Table II shows that, following transplantation of the duodenum into the small bowel well away from the discharge of unaltered stomach content, there was an increase in output of free HCl which ranged from 113% to 364%. In this group, the difference between pre-operative and post-operative means was also found to be significant.

Fig. 1 and 2 demonstrate graphically the increase in pouch secretion following the two operations; and they also show the variation in output of the Heidenhain pouches from day to day—a variation shown by all dogs in both groups both pre-operatively and post-operatively. The increase in mean output following the operations was accompanied by an increase in variability of output; the increase between mean and the standard deviation was roughly linear.*

Discussion. The magnitude of increase in output of free HCl from dogs with Heidenhain gastric pouches following both duodenectomy and duodenal transplantation is indeed impressive. The findings, however, should not come as a great surprise, inasmuch as Sokolov(1) reported that when fat, 0.5% HCl or gastric juice were introduced into the duodenum of Pavlov-pouch dogs with gastric and duodenal fistulas, both amount and digestive ability of the juice secreted by the pouch were reduced.

There is no indication in these experiments as to the mechanism in the duodenum responsible for the tremendous increase in gastric secretion in these dogs. Presumably it is the same in dogs following duodenectomy as well as after duodenal transplant. Delay in gastric emptying time could be responsible, in that an increase in the chemical phase of gastric secretion would result from food remaining in contact with the antral mucosa for longer periods, thereby causing an increase in amount of gastrin liberated. The possibility of such an explanation being a likely cause was ruled out by submitting one dog from each group to a barium meal which was followed fluoroscopically. Gastric emptying and progress through the small bowel were normal; and there appeared to be no delay in emptying of stomach or transit of barium beyond the anastomoses; however, no pre-operative studies were made upon dogs prior to operative procedures.

Fat has been known to exert its inhibitory effect on gastric secretion only after it enters the small bowel, particularly the duodenum, since the work of Sokolov in 1904. Farrell and Ivy(4) and Feng, Hou and Lim(5) proved that this mechanism was humoral when they demonstrated the same effect in auto-transplanted fundic pouches completely separated from their original blood supply. Kosaka and Lim(6) extracted the hormone from the duodenal mucosa and named it "enterogastrone." Hydrochloric acid and gastric juice were shown by Sokolov(1) to cause an inhibition of gastric secretion when introduced into the duodenum. This finding was confirmed by Day and Webster(7) and by Pin-

* No attempt was made to get a pooled estimate of variance as is appropriate in Student's "t" test, since there was an increase in variance corresponding to the operation. In results presented, the difference in means is, in every case, sufficiently large with respect to its standard error that the approximate test may be regarded as conclusive.

cus, Thomas, and Rehfuss(8) who found an inhibition of gastric secretion from an isolated gastric pouch stimulated by a test meat meal when either N/10 HCl or gastric juice were introduced into the duodenum. Neutralized gastric juice had no effect. The mechanism of the action of acids may be hormonal, since the dogs of Day and Webster did not have innervated pouches. Sugars were also shown to have an inhibitory effect on gastric secretion when introduced into the duodenum by Day and Komarov(9). This inhibitory effect was stronger when glucose was introduced directly into the duodenum than when the same amount was given intravenously. The degree of inhibition varied directly with the concentration of glucose used, and Day and Komarov concluded that its inhibitory action was probably due to its osmotic effects. Peptones and products of protein hydrolysis have been shown by Thomas and Crider(10) to have an inhibitory effect on gastric motility when placed in the duodenum; however no inhibitory effect of these substances on gastric secretion has been reported.

It is possible that the peptic ulcers which follow the Mann-Williamson procedure, as well as those which follow the modified Mann-Williamson procedure as described by Keefer, Martin and Glenn(3) may be due, at least in part, to augmentation of gastric secretion attending exclusion unaltered gastric contents away from duodenal mucosa. This occurrence also may help to account for the increase in incidence of stomal ulceration attending gastric resection in which a long afferent duodenojejunal loop is employed in gastrojejunostomy over that observed following employment of a short afferent loop as reported by Merendino *et al.*(11,12).

Summary. 1. The output of free HCl by isolated gastric pouches more than doubled following resection of the duodenum and proximal 20 to 30 cm of jejunum. 2. Interposing a segment of small bowel approximately 1.5 meters long between the stomach and duodenum also caused the output of free HCl from isolated gastric pouches to more than double. 3. The marked increase in HCl secretion following both operations was observed in every case and seemed to be independent of the pre-operative level of HCl secretion by the pouches.

1. Sokolov, A. P., *Jahresber. Fortschr. Tierchem.*, 1904, v34, 496.

2. Storer, E. H., Oberhelman, H. A., Woodward, E. R., Smith, C. A., and Dragstedt, L. R., *A. M. A. Arch. Surg.*, 1952, v64, 192.

3. Keefer, E. B. C., Martin, K. A., and Glenn, F., in *Surgical Forum,* 1953, W. B. Saunders Co., Philadelphia, 1954, p330.

4. Farrell, J. I., and Ivy, A. C., *Am. J. Physiol.*, 1926, v76, 227.

5. Feng, T. P., Hou, H. C., and Lim, R. K. S., *Chinese J. Physiol.*, 1929, v3, 371.

6. Kosaka, T., and Lim, R. K. S., PROC. SOC. EXP. BIOL. AND MED., 1930, v27, 890.

7. Day, J. J., and Webster, W. D., *Am. J. Digest. Dis.*, 1935, v2, 527.

8. Pincus, I. J., Thomas, J. E., and Rehfuss, M. E., PROC. SOC. EXP. BIOL. AND MED., 1942, v51, 367.

9. Day, J. J., and Komarov, S. A., *Am. J. Digest. Dis.*, 1939, v6, 169.

10. Thomas, J. E., and Crider, J. O., *Am. J. Physiol.*, 1939, v126, 28.

11. Merendino, K. A., Varco, R. L., Litow, S., Kolauch, F., Baronofsky, I., and Wangensteen, O. H., PROC. SOC. EXP. BIOL. AND MED., 1945, v58, 222.

12. Merendino, K. A., Lanin, B. G., Kolouch, F., Baronofsky, I., Litow, S., and Wangensteen, O. H., *ibid.*, 1954, v58, 226.

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Spontaneous Perforation of the Esophagus; Experimental Study. (21571)

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The cause of sudden esophageal perforation which occurs in the absence of any recognizable pre-existing esophageal disease or symptoms has been the subject of considerable speculation and controversy. The thesis that esophageal perforation might be due to the increased hydrostatic pressure within the esophageal lumen associated with the act of vomiting has been favored by many writers. This increased pressure could stretch and tear the esophageal wall. Mallory and Weiss(1) found that if a human stomach were partly filled with water and squeezed while the pylorus was obstructed and the esophagus partly constricted at the level of the diaphragm, vertical linear tears were produced in the mucosa of the lower esophagus which extended down into the cardia of the stomach. The bursting pressure of the human esophagus was measured by Kinsella, Morse, and Hertzog(2) in cadavers. They found that this pressure varied between 100 and 300 mm of mercury, with an average of 193 mm of mercury in 29 subjects. These values agree closely with those reported by Mackenzie(3) and by Burt(4). When the esophagus is ruptured by air or water pressure, the point of rupture is always in the lower third of the esophagus and always appears as a linear slit or tear. The bursting pressure of the human esophagus approaches the physiological range, for Kinsella points out that an average adult can, by forced expiration, blow with a pressure of 110 to 125 mm of mercury, and that a strong man can sometimes attain a pressure of 180 to 190 mm of mercury. On the other hand, the bursting pressure of both the dog and cat esophagus, as reported by Ferguson and his associates(5) from this laboratory, is far beyond the physiological range; yet, as will be pointed out later, esophageal perforations quite similar to the spontaneous perforations seen in humans can be produced experimentally in these animals without the use of high pressures. Ferguson found that the average bursting pressure of the normal dog esophagus was 830 mm of mercury and that of the cat esophagus was in excess of 1200 mm of mercury. There is considerable evidence that regurgitation of acid peptic juice into the esophagus may be the primary cause of acute esophageal perforation. The extreme susceptibility of the esophagus to injury by digestive juices has been shown in several ways at this clinic. Arroyave, Clatworthy, and Wangensteen(6) showed that a tiny island of gastric mucosa transplanted into the esophageal wall invariably produced severe esophageal ulceration. Cross and Wangensteen(7) showed that bile and pancreatic juice, when diverted into the esophagus of dogs, produced severe esophageal erosions. Ferguson and his associates(5) reported a large series of experiments on the effects of acid-peptic juice on the esophagus. They perfused the esophagus of living cats with canine gastric pouch juice, and found that esophageal perforation occurred in one instance in as brief an interval as 19 minutes following commencement of the perfusion. In a series of 12 cats, the minimum perfusion time before perforation occurred was 19 minutes and the maximum time was 2.3 hours. The perfusion pressure was only 20 cm of water, so that hydrostatic pressure was a negligible factor. Perfusion of the cat esophagus with hydrochloric acid at pH 1 did cause esophagitis, and in some animals, esophageal perforation. But the perforations occurred only after much longer periods of time (5 to 11 hours). It should be mentioned that the perforations resulting from these perfusion experiments occurred in the lower third of the esophagus and were linear, having the appearance of linear slits or tears. Ferguson *et al.*(5) also reported a series of experiments in which repeated vomiting was produced in cats and dogs by obstruction of the pylorus. Intramuscular histamine-in-beeswax injections were given to assure maximal secretion of highly acid gastric juice. In a group of 13 dogs treated in this way, 4 developed esopha-

geal perforations, 2 more had severe esophagitis, 4 had moderate esophagitis, and 3 had slight esophagitis.

The present study was undertaken to extend these observations and to furnish additional control data.

Methods. Mongrel dogs were anesthetized with intravenous pentobarbital sodium in a dose of approximately 30 mg per kilo. The abdomen was opened in the midline and the pylorus isolated and doubly ligated with heavy cotton twine; the abdomen was then closed. The animals were allowed to recover for about 16 hours, and were then given daily intramuscular injections of 30 mg of histamine base as a histamine-in-beeswax mixture. Fluid and electrolyte losses were replaced with intravenous infusions of 5% dextrose in Ringer's solution. The dogs were divided into 4 groups. Group 1 had the pyloric ligation alone, and the other groups were subjected to additional operative procedures. Group 2 had a complete excision of the acid-secreting area of the stomach with an end-to-end esophagoantrostomy approximately 4 weeks prior to pyloric ligation. This operation was done so that there would be no acid-peptic juice passing through the esophagus when the dog vomited. Group 3 had a double-barrelled cervical esophagostomy done prior to the pyloric ligation. This was done to prevent swallowed saliva from reaching the distal portion of the esophagus. Group 4 had both the complete excision of the acid secreting area of the stomach and the double-barreled cervical esophagostomy done prior to the pyloric ligation. An additional experiment was done to determine the intraluminal pressure in the esophagus and stomach during vomiting and to compare these pressures with the intrathoracic pressure. Small water-filled rubber balloons were placed in the stomach and esophagus and an open catheter was placed in the closed thorax. Pressures were measured continuously and simultaneously with Statham strain gauges and recorded with a Sanborn Polyviso recorder. Vomiting was produced in the dogs as a result of injections of apomorphine or by over-distention of the stomach with a dilute solution of copper sulfate instilled through a gastric tube. The dogs were fed prior to the experiment so that the vomitus would be more like that accompanying spontaneous vomiting. Four dogs were used in this part of the study; 3 of these had pyloric ligation alone, and 1 had a complete excision of the acid-secreting area of the stomach prior to pyloric ligation.

Results. The maximal simultaneous pressures recorded during the act of vomiting in the 4 dogs tested were: intra-esophageal—136 mm Hg; intra-gastric—100 mm Hg; and intra-thoracic—60 mm Hg. The pressure readings in the dog in which excision of the acid-secreting area had been done were essentially the same as those in the dogs with intact stomachs.

The results of the remainder of the experiments are summarized in Fig. 1. In Group 1 (pyloric ligation alone) 2 out of 20 dogs developed esophageal perforations, and died 4 and 6 hours respectively after the first injection of histamine. Ten more dogs in this group developed a severe degree of esophagitis, making a total of 12 dogs (60% of the group) with severe esophagitis.

In Group 2 (pyloric ligation following excision of the acid-secreting area of the stomach) no esophageal perforations, and indeed, no esophagitis occurred in the entire group of 7 dogs. These dogs survived an average of 7 days following the first injection of histamine, with a range of 3 days to 17 days.

In Group 3 (pyloric ligation following double-barrelled cervical esophagostomy) there was a far higher incidence of esophageal perforation than in Group 1 (pyloric ligation alone). Ten dogs out of 25 developed esophageal perforations. These perforations occurred in from 4 to 18 hours after the first injection of histamine. Nine more dogs had a severe degree of esophagitis, making a total of 19 dogs (76% of the group) with severe esophagitis.

Group 4 (pyloric ligation following excision of the acid secreting area plus double-barrelled cervical esophagostomy) had no esophageal perforations and no esophagitis. The average survival for the 4 dogs in this group was 9 days following the first histamine

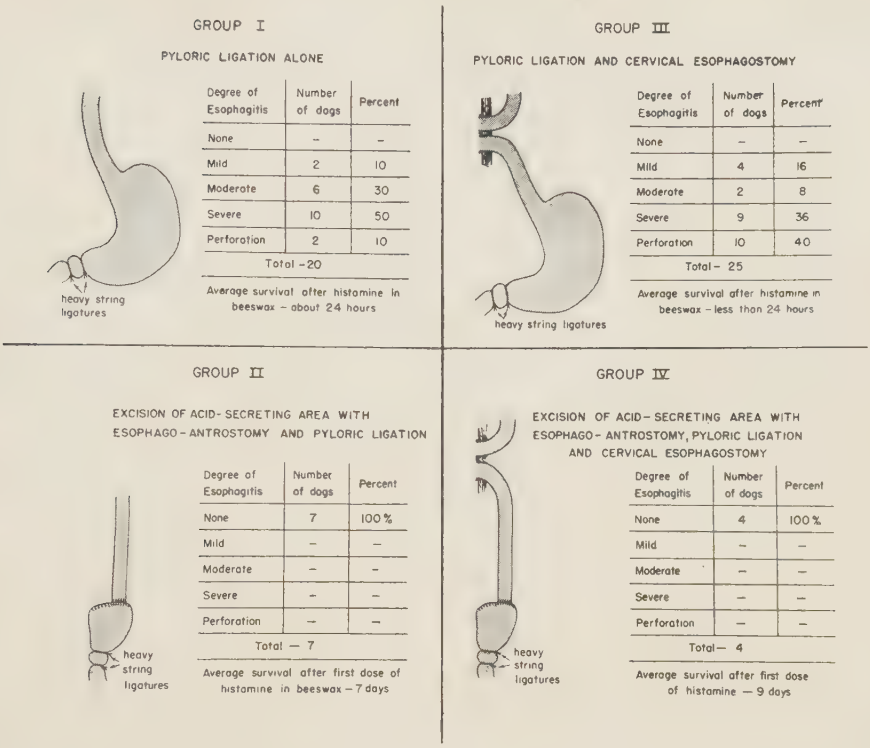

FIG. 1. Effect on esophagus of repeated vomiting caused by obstruction of gastric outlet following various operative procedures. Each dog was given a daily intramuscular dose of histamine-in-beeswax to assure repeated emeses of a highly acid gastric juice.

injection. The range of survival was 7 to 13 days.

The most common site of the esophageal perforations which occurred in these experiments was on the left lateral wall of the upper third of the thoracic esophagus. The perforations were, without exception, elongated in the longitudinal axis of the esophagus and had the appearance of linear slits or tears. In most instances, there was a severe degree of generalized esophagitis with necrosis of much of the mucosa of the upper thoracic and lower cervical esophagus associated with the esophageal perforation. However, in some instances, these changes were limited to the esophageal mucosa immediately surrounding the perforation. In every case, the perforation was associated with a severe mediastinitis and a large collection of cloudy, dark brown fluid and some air in one or both pleural cavities. Often the supporting tissues of the posterior mediastinum were completely digested away, leaving the major vessels and nerves cleanly exposed as if they had been carefully dissected out in an anatomical study. The mediastinitis and empyema found in these dogs, in the light of the circumstance that many of them were autopsied within 5 minutes after death, indicate that the perforations occurred prior to death and were not post-mortem changes.

Microscopic examination of the esophageal wall in the region of these perforations usually revealed large areas of superficial ulceration in which the squamous lining was denuded. There was a superficial zone of necrosis and the underlying submucosa was diffusely infiltrated with lymphocytes and polymorphonuclear leukocytes. The inflammatory reaction did not usually involve the muscular layer of the esophageal wall to any great extent, but the adventitia often contained a diffuse inflammatory exudate as a result of the severe mediastinitis and pleuritis following the perforation.

Discussion. The changes seen in both the

gross and microscopic examinations of the esophageal perforations of the dogs in these experiments have been observed to a greater or lesser degree in most of the human case reports where detailed gross and microscopic descriptions have been included. Of course, the extensive necrosis of almost the entire esophageal mucosa with marked brownish black discoloration has not been described; however, local areas of brownish black necrosis have been seen in human cases. In fact, Higgenson and Clagett(8) reported a case where there was complete dissolution of the entire circumference of the esophageal wall for a short distance above the diaphragm. The perforation in this case followed repeated retching and vomiting while the patient was on the operating table under spinal anesthesia.

It seems clear from the experimental data presented here that increased hydrostatic pressure within the lumen of the esophagus played only a very minor role in the production of the esophageal perforations seen in these dogs, if, indeed, it played any role at all. It is also clear that acid-peptic injury to the esophageal wall was the important factor in their production. The most conclusive proof of this is the fact that excision of the acid-secreting area of the stomach entirely prevents esophageal injury following ligation of the pylorus. None of the dogs in Groups 2 and 4 where the acid-secreting area was excised developed esophageal perforations, and in fact, none developed even a mild esophagitis.

The extremely high incidence of esophageal perforation in the dogs in Group 3 (pyloric ligation following cervical esophagostomy) as compared with Group 1 (pyloric ligation alone) is also presumptive evidence that acid-peptic digestion is important in causing these perforations. Diversion of the swallowed saliva away from the distal esophagus robs it of part of its mucoid protective coat and of the neutralizing effect of the saliva on gastric juice.

Summary. 1. Esophageal perforations, similar in many respects to the so-called "spontaneous" perforations of the esophagus seen in humans, can be caused in dogs by ligation of the outlet of the stomach followed by intramuscular administration of histamine-in-beeswax. 2. The incidence of esophageal perforation is far higher following pyloric obstruction in dogs that have a double-barrelled cervical esophagostomy prior to pyloric ligation. Presumably this is due to the loss of the protective effect of the saliva. 3. Excision of the acid-secreting segment of the stomach not only prevents esophageal perforation following pyloric ligation, but also completely prevents the development of any esophagitis.

1. Mallory, G. K., and Weiss, S., *Am. J. Med. Sc.,* 1929, v178, 506.

2. Kinsella, T. S., Morse, R. W., and Hertzog, A. S., *J. Thor. Surg.,* 1948, v17, 613.

3. Mackenzie, M., *Diseases of the Nose and Throat,* v2, 1884, Blakiston Co., Philadelphia, p169.

4. Burt, C. A. V., *Arch. Surg.,* 1931, v22, 875.

5. Ferguson, D. S., Sanches-Palomera, E., Sako, Y., Clatworthy, H. W., Toon, R. W., and Wangensteen, O. H., *Surgery,* 1950, v28, 1022.

6. Arroyave, R., Clatworthy, H. W., and Wangensteen, O. H., *Surgical Forum,* 1950, W. B. Saunders Co., Philadelphia, 1951, p57.

7. Cross, F. S., and Wangensteen, O. H., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 862.

8. Hegginson, J. F., and Clagett, O. T., *J. Thor. Surg.,* 1948, v17, 846.

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Pantethine and Casein Hydrolyzate in the Growth of Certain Lactobacilli. (21572)

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Veltre *et al.*(1) and Shorb and Veltre(2) observed that *Lactobacillus bifidus* of avian origin could not be grown on a completely synthetic medium but required certain natural materials for optimum growth. Studies of nutritional requirements of some rumen bacteria in the present investigation indicated that they also required unidentified factors having a similar distribution in natural materials. This report describes the requirements of some rumen lactic acid bacteria and includes an observation on the nutrition of avian *L. bifidus*.

Methods. The basal medium was either that used in the assay of vit. B_{12} with *Lactobacillus leichmannii*(3) or that of Shorb and Veltre(2). The cultures used were RO-H_2 described by Huhtanen and Gall(4), RO-CR and RO-L_5 described by Huhtanen and Gall (5). The avian bifid type was the same strain used by Shorb and Veltre(2). The medium for RO-H_2 contained glucose as the carbohydrate since this organism was not able to utilize lactose; conversely, the other types utilized glucose poorly or not at all but responded well with lactose as energy source. All cultures were routinely carried in the complex organic broth of Huhtanen *et al.*(6). Stock cultures were stored at room temperature and retained viability for at least 2 months without transfer. Inoculation was made by needle directly from a 24-hour culture. This method of inoculation was necessary due to the difficulty of centrifuging the tiny cells of RO-H_2 and to the sensitivity to oxygen of rumen anaerobes. The enzyme-hydrolyzed casein was prepared by digesting 100 ml of a 2% solution of Difco vitamin-free casein in 1% Na_2HPO_4 at pH 8.8 for 4 hours at 37°C with 50 mg of Difco pure trypsin.

Results. No growth was obtained in the B_{12} assay medium with RO-L_5, RO-CR or RO-H_2 with or without added B_{12}; however, good growth was obtained by addition of graded amounts of yeast extract or whole brewers' yeast. Certain other substances including hot water extracts of plant materials, Phytone and distillers' solubles, all showed activity. An acetone extract of a propionibacterium-like microorganism, RO-c1, described by Huhtanen and Gall(5) also showed good growth-promoting activity. Growth equal to that with yeast extract was obtained with a mixture of enzyme-hydrolyzed casein and the synthetic form of *Lactobacillus bulgaricus* factor (LBF, pantethine). No further stimulatory factors could be demonstrated. Pantothenic acid in concentrations up to 50 γ/ml would not replace pantethine. Coenzyme A of 3 different purities 50%, 70% and 95% produced growth responses in proportion to the amount of CO A present indicating that the bacteria responded to both the free and combined forms of LBF.

A culture of the avian bifid in Shorb's medium was also found to respond to enzyme-hydrolyzed casein and pantethine but a more marked response was obtained from hydrolyzed casein than was obtained in the case of the rumen bacteria. Table I shows that this combination produced a growth response equal to that obtained from fish solubles. The fish solubles, however, greatly accelerated growth with good growth apparent within 24 hours while with the pantethine plus enzyme hydrolyzed casein mixture at least 2 days were required for growth to develop. The pantethine requirement of the avian bifid was also replaceable by coenzyme A but not by excess pantothenic acid. At least 60% of the response to fish solubles could be replaced by pantethine alone, the remainder from some factor in hydrolyzed casein. This factor was not B_{12}, orotic acid or thioctic acid.

It seemed of interest to determine the possible relationship of the avian bifid to RO-L_5 and RO-CR. The observation was previously

TABLE I. Response of Avian *Lactobacillus bifidus* to Pantethine and Trypsin Hydrolyzed Casein. Results in terms of titratable acidity (ml 0.1 N NaOH per tube).

Material assayed	Other Additions	ml assay material/tube .5	1.0	5.0
Fish solubles	None	3.9	5.5	6.0
Pantethine (0.58 γ/ml)	"	4.6	4.2	4.3
Idem	Trypsin hyd. casein	5.4	5.4	5.3
"	B_{12}	4.2	4.2	4.5
"	B_{12}, orotic acid	4.0	4.3	4.2
"	B_{12}, orotic acid, thioctic acid	4.5	4.5	4.3
"	B_{12}, orotic acid, thioctic acid, trypsin hyd. casein	5.8	5.7	5.4

With no additions titratable acidity was 0.5 ml 0.1 N NaOH. Fish solubles—5% solution steamed and centrifuged. B_{12} 0.2 γ/tube, orotic acid 8 γ/tube, thioctic acid 1.6 γ/tube. Trypsin hyd. casein 10 mg/tube. All solutions neutralized. Final volume 10 ml.

made by Huhtanen and Gall(5) that resting cells of RO-L_5 and RO-CR utilized starch and maltose more rapidly than glucose, a phenomenon not observed with any other lactic acid bacteria. The avian bifid was accordingly grown in the rich organic medium for 24 hours, the cells were centrifuged, washed and resuspended in 0.9% $NaHCO_3$ previously gassed with CO_2 to pH 6.8. The manometric response to glucose, maltose and starch showed that starch and maltose were more readily utilized than glucose. The morphology of the avian bifid was different from that of RO-L_5 which showed no bifurcate rod forms, but RO-CR has been observed to grow in such arrangement. The response of RO-H_2 to these carbohydrates was not determined manometrically.

Discussion. The Lactobacilli studied in this report do not appear to have unusual nutritive requirements other than the requirement for maltose, lactose or starch. Gyorgy *et al.*(7) also indicated that *Lactobacillus bifidus* and *L. bifidus* var. *Penn* ferment lactose or maltose more readily than glucose. This property appears to differentiate bifids from other Lactobacilli and may serve to differentiate them in cases where morphology alone is an insufficient criterion.

The inability of Shorb and Veltre to demonstrate the requirement of the avian bifidus for pantethine may be because they used a different form of LBF. Long and Williams (8) showed that there were several chromatographic entities related to LBF which exhibited growth stimulating properties for Lactobacilli.

Addendum. Since preparation of this manuscript, Dr. M. S. Shorb has kindly repeated previous work with the finding that one strain of avian bifidus responds as indicated in this report but that freshly isolated strains may not show the same response to pantethine and enzyme hydrolyzed casein.

Summary. Pantethine and enzyme-hydrolyzed casein were found to supplant crude natural materials as sources of a presumed unidentified growth factor for several rumen bacteria and for avian *Lactobacillus bifidus*.

The author is indebted to Dr. E. E. Snell, University of Texas for the pantethine, Dr. F. M. Strong, University of Wisconsin, for the samples of Co A and Dr. M. S. Shorb, University of Maryland, for the culture of avian *Lactobacillus bifidus*.

1. Veltre, F. A., Shorb, M. S., and Pelczar, M. J., Jr., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 284.

2. Shorb, M. S., and Veltre, F. A., *ibid.*, 1954, v86, 140.

3. Pharmacopeia of the United States, 3rd Suppl., XIV, Revision, 1951, v15.

4. Huhtanen, C. N., and Gall, L. S., *J. Bact.*, 1953, v65, 548.

5.———, *ibid.*, 1953, v65, 554.

6. Huhtanen, C. N., Rogers, M. S., and Gall, L. S., *ibid.*, 1952, v64, 17.

7. Gyorgy, Paul, Norris, Robert F., and Rose, Catherine, *Arch. Biochem. and Biophysics*, 1954, v48.

8. Long, Calvin L., and Williams, William L., *J. Bact.*, 1951, v61, 195.

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Growth of Vaccinia Virus on Mouse Ascitic Tumor Cells *in vivo*.* (21573)

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The importance of vaccinia as a laboratory virus warrants the attempt to grow it in a host-virus system similar to that developed by Ackermann and Kurtz in their adaptation of influenza virus to growth in the Ehrlich ascites tumor cells of mice *in vivo*(1). Moreover, it seemed possible that an *in vivo* adapted strain might be more amenable to growth in ascites tumor cells cultured *in vitro* than would an unadapted strain, a possibility which was considered after failure on our part to grow the virus directly in the tumor cells cultured *in vitro* by the usual technics. In the present preliminary report (confined to the *in vivo* experiments), the adaptation of vaccinia virus to culture in the ascitic form of 2 mouse tumors is described.

Materials and methods. Tissues. Mouse sarcoma 37 cells capable of being propagated in the ascitic form were obtained by mashing solid tumor tissue through a metal screen of ½ mm mesh. The washed tumor cells (separated by vigorous pipetting in 0.85% NaCl solution) were injected into the peritoneal cavities of mice. The tumor was then maintained in ascitic form by withdrawing ascitic fluid from mice which had been inoculated with the tumor cells 3 to 5 days previously, 4,000,000 to 10,000,000 tumor cells in 0.3 ml of 0.85% NaCl solution being injected intraperitoneally into each of 5 mice for every passage. Krebs tumor (normally an ascitic form) was propagated in the same manner. *Virus.* The WR-1 (Western Reserve) strain of vaccinia virus which had been propagated in the mouse brain but which possessed dermal affinity for the rabbit was used in these experiments. *Virus titer.* Viral titers were calculated by the standard technic of intradermal injection of rabbits and by intracerebral injection of young mice. Data obtained previously in this laboratory had shown that the 50% infectivity titer obtained by the latter method was one 10-fold dilution less than that obtained by intradermal injection of rabbits.

Results. Sarcoma-vaccinia experiments. Three or 4 mice bearing 3- or 5-day-old sarcoma 37 ascitic tumors were inoculated intraperitoneally with 0.2 ml of a 10^{-1} dilution of an homogenate of mouse brain infected with vaccinia WR-1 virus (possessing a virus titer on the rabbit skin of $10^{-3.5}$/ml). After 3 days, peritoneal fluid (2 to 5 ml from each mouse) was withdrawn, pooled, centrifuged to sediment the cells, and 0.3 ml of the supernatant inoculated intraperitoneally into each of 3 or 4 tumor-bearing mice for the next sarcoma-vaccinia passage. A portion of the supernatant was sealed in glass and stored at –70°C for later titration on the rabbit. Every 3rd day the procedure was repeated. Titration of the virus content of the first 6 passages and of the original mouse brain suspension was then carried out by intradermal injections of the rabbit. Typical lesions which progressed to the hemorrhagic state on the 5th day after injection developed at the sites of injection of the original mouse brain material and of the first 2 passages of peritoneal fluid. Later passages caused inflammatory reactions by the 3rd day after injection, but these lesions did not progress to the hemorrhagic state thereafter, it appearing that the virus had partly lost its dermal affinity for the rabbit by repeated passages in the mouse. All lesions considered positive for purposes of virus titration were definite and sufficiently distinct to be read on the 5th day. The infectivity titer of the first passage was $10^{-1.2}$ but had fallen to zero (*i.e.*, was below the sensitivity threshhold of the titration method) by the 3rd passage, the 4th passage had a titer of $10^{-0.5}$, the 5th passage $10^{-1.6}$, and the 6th passage had a titer of $10^{-4.5}$. A second series of experiments was carried out exactly as the first series, except that the infected peritoneal fluid was homogenized in an attempt to break up the tumor cells

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and release the virus. The cell-free supernatant was used to make further passages in tumor-bearing mice. In addition, 0.03 ml of the undiluted supernatant of each passage was immediately injected intracerebrally into each of 3 or 4 mice to test for the presence of virus. Cerebral symptoms developed on the 2nd or 3rd day, 90% of the mice (28) died on the 4th or 5th day, and the remainder were dead by the 7th day. This was true for every passage from the 1st through the 10th. The 9th passage material was titrated in the mouse brain in 10-fold dilutions, yielding a titer of $10^{-3.5}$ (roughly equivalent to $10^{-4.5}$ on the rabbit). *Krebs-vaccinia experiments.* After 5 serial passages of vaccinia in the peritoneal cavities of mice bearing Krebs ascitic cells, an intradermal titration of each passage was performed on a single rabbit. The 1st passage had a titer of $10^{-2.5}$, the 2nd passage a titer of 10^{-1}, the virus was not detectible on the 3rd passage, the titer of the 4th passage was $10^{-0.8}$, and that of the 5th was $10^{-1.5}$. The lesions on the rabbit's skin caused by the later passages were inflammatory rather than hemorrhagic but were quite distinct on the 5th day after injection. A second series of passages, carried out in the same way as the first series (except that the peritoneal fluid of each passage was homogenized before starting another passage) showed, upon intracerebral inoculation of mice, that the virus was definitely present in the peritoneal fluid of each passage, from the 1st through the 10th passage. In the present experiments, however, all mice receiving intracerebral injections survived until the 8th day and a few until the 23rd day (in contrast to death not later than the 7th day after injection of the sarcoma 37 adapted virus). Beginning with the 4th passage all of the mice (26) developed cerebral symptoms and died between the 10th and 15th days. *Influence of vaccinia virus on ascites tumor cells.* To determine whether or not tumor cells in the peritoneal cavities of the mice remained viable after coming into contact with the virus, undiluted peritoneal fluid taken 3 days after the injection of the virus from one of the sarcoma-vaccinia passages and containing many tumor cells was injected intraperitoneally into 5 normal mice. These mice appeared healthy on the 7th day, when they were sacrificed and 1 ml of 0.85% NaCl solution added to the peritoneal cavity of each. The fluid from each mouse was examined microscopically and found to contain the cells normally found in the peritoneal cavity but few or no tumor cells, indicating that most of the tumor cells had failed to survive. Autopsies failed to disclose gross lesions of the gut or the presence of solid tumorous masses. *Controls.* To make certain that non-viral toxic material in the peritoneal cavity was not causing death of the mice upon intracerebral inoculation, peritoneal fluid from tumor-bearing mice (not previously infected with the virus) was harvested on the 3rd day after inoculation of the tumor, homogenized, centrifuged, and injected intracerebrally into 5 normal mice, all mice surviving beyond the 30th day. Since vaccinia virus might conceivably propagate on mouse peritoneum as it is reported to do on rabbit peritoneum(2,3), a heavy initial injection of the original vaccinia-infected mouse brain suspension was administered intraperitoneally to a new lot of mice. A total of 4 passages (at 3-day intervals) was completed, the peritoneal fluid from each passage being tested intradermally in rabbits and found not to contain virus. Thus the virus did not grow in the absence of the tumor cells.

Discussion. The above experiments demonstrate that vaccinia virus can be grown on ascites tumor cells in the peritoneal cavities of mice, since in each of 4 separate series of serial passages (involving 5 to 11 passages in each series and a total of 31 passages), the virus was found to be present after the final passage, although approximately a 10-fold dilution of the original inoculum was made at each passage. The pattern of adaptation was similar to that observed by Ackermann and Kurtz in their studies on the influenza virus (the virus titer dropping during the first 3 passages and then rising consistently thereafter). Loss of viability of the tumor cells, associated with the multiplication of the virus in the present experiments, was likewise in keeping with a similar effect of virus on host cells noted by Ackermann and Kurtz. The fact that vaccinia virus can be grown in two widely different kinds of tumor cells, selected

at random in the present experiments, indicates that the method might be of wide application, particularly since a solid tumor (sarcoma 37) was made to yield to the method.

Summary. Adaptation of vaccinia WR-1 virus to growth on 2 ascitic forms of mouse tumors (sarcoma 37 and Krebs) has been accomplished by means of serial passages in the peritoneal cavities of mice. Loss of viability of the tumor cells was associated with multiplication of the virus.

The author wishes to thank Drs. F. K. Sanders and G. N. C. Crawford for helpful suggestions and assistance in carrying out these experiments and Prof. A. C. Hardy for permitting the use of laboratory space in Dept. of Zoology and Comparative Anatomy, Oxford University.

1. Ackermann, W. W., and Kurtz, H., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 421.

2. Douglas, S. R., Smith, W., and Price, L. R. W., *J. Path. Bact.*, 1929, v32, 99.

3. Borrel, A., *C. R. Soc. Biol.*, Paris, 1936, v121, 1084.

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Mammary Tumor Regression by Extracts of Histolyzed Amphibian Skin and Gills. (21574)

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In 1948 the writer obtained complete regression of spontaneous mammary tumors of mice by the periodic injections of neutralized acid extracts of degenerating tail skin obtained from involuting *R. catesbeiana* larvae. This finding represents the first and only instance in which extracts of an amphibian tissue have been found effective in bringing about the total degeneration of actively growing spontaneous mammary tumors of mice. The present work represents a continuation of these results as regards the action of skin extracts and also relates the effects of extracts derived from degenerating amphibian muscle and gill tissues.

Materials and methods. The extract of tail skin from normally metamorphosing *R. catesbeiana* larvae was of the same lot as previously prepared, used, and described by the writer (1). It was now approximately 15 months old, however, and its use made possible an evaluation of the possible continued potency of its tumor destructive qualities. Two new extracts were also utilized; one from histolyzing larval tail muscle and the other from degenerating larval gills. Both these preparations consisted, like the original skin extracts previously described(1), of neutralized HCl extracts. There were two points of difference, however, in that the muscle and gill tissues were obtained from *R. clamitans* larvae which were undergoing *artificially* induced metamorphosis. The effect of injecting sterile distilled water was also determined and, like other experimental series, the results were checked against those of tumors injected with neutralized HCl control solutions. Mice with rapidly growing spontaneous mammary tumors were supplied by Carworth Farms, New City, N. Y. All extracts and control solutions were injected at 2 day intervals directly into the tumors for a total of 6 injections per animal. The dosage at each injection was 0.15 cc.

Results. The results of the skin extract injections are listed in Table I. Only those animals which lived for more than 10 days following the last injection were tabulated. The results of the skin extract injections showed that a 30.7% average reduction in length plus breadth dimensions of the tumors had taken place within 12 days following the first injection. One tumor regressed completely within 12 days and 4 (50%) of the others regressed later. The control tumors showed only a 5.0% average reduction within this same time limit while none underwent subsequent complete degeneration. Since the ratio of the difference between these 2 means to the probable error of the difference was 3.72, the results are

TABLE I. Effect of Various Agents on Spontaneous Mammary Gland Tumors of Mice.

Treatment	No. in group	Tumor size* (mm) †			
R. catesbeiana					
Skin extr.	9	24/25 (20/22), 25/21 (13/26), 38/31 (21/26)	34/30 (19/26), 27/18 (0/58),	38/ 0 (0/53), 31/22 (0/56),	26/13 (0/53), 26/20 (0/61),
Control sol.	8	23/23 (25/50), 24/21 (18/66),	21/26 (23/57), 35/32 (29/26),	34/30 (23/23), 31/28 (25/51),	33/29 (24/20), 33/30 (24/59)
R. clamitans					
Muscle extr.	9	28/20 (15/61), 33/40 (44/29), 26/26 (33/29)	27/18 (13/66), 40/39 (42/29),	30/29 (26/62), 38/42 (54/54),	20/23 (29/53), 35/29 (24/57),
Gill extr.	11	23/13 (0/57), 37/30 (17/27), 23/ 9 (0/60),	31/28 (14/27), 26/17 (0/63), 27/18 (0/27),	24/ 9 (0/52), 38/34 (21/27), 36/15 (7/58)	28/ 8 (0/59), 19/16 (0/61),
Control sol.	10	30/31 (30/52), 39/42 (33/55), 26/24 (28/62),	23/23 (20/30), 22/25 (28/58), 31/30 (29/57)	39/29 (21/27), 37/43 (53/23),	27/22 (19/30), 32/29 (22/51),
Distilled water	5	36/40 (48/64), 26/32 (39/25)	27/33 (34/25),	33/33 (44/52),	42/48 (52/57),
Control sol.	4	35/30 (25/25),	28/30 (25/58),	40/36 (36/25),	32/34 (28/60)

* Initial tumor size over size 12 days after first treatment. In parentheses, size over days after first treatment when animal died or was killed for autopsy.

† All survival times 20-30 days, inclusive, were animals killed for autopsy; 50-66 days, inclusive, animals died.

statistically significant in that the 15 months old skin extract was apparently still potent, although much less so since, when first used (15 months previously), the results showed a ratio of 7.42. The wide variation between these 2 ratios may not, however, be a true measure of the degree of potency drop, so indicated, when one considers that the earlier ratio of 7.42 was based on but 3 injected and 3 control tumors, whereas the present ratio of 3.72 was calculated from the results of 9 injected and 8 control cases.

The results of the tail muscle extract injections are listed in Table I. Although the average length plus breadth tumor reduction (4.2%) within 12 days following the first injection was greater than that of the controls (2.4%), this difference was of no statistical significance since the ratio of the difference between the 2 means to the probable error of their difference was but 0.35. In fact, it will be noted that the per cent reduction of the experimental tumors (4.2%) was even slightly less than that of the control group (5.0%) used for the skin extract series. It seems evident, therefore, that the muscle extracts possessed no additive degenerative effects as compared with the control solutions. In this respect, *R. clamitans* tail muscle extracts correspond to previously obtained but unpublished results of the writer when using *R. catesbeiana* tail muscle extracts.

The results of the gill extract injections are listed also in Table I. Here, in contrast to the results of muscle extract injections, a pronounced degenerative effect on the tumors is to be noted amounting to an average reduction of 38.1% within 12 days following the first injection as compared with but 2.4% for the controls; the same controls being used for this series as for the muscle extract series. The marked ratio of 6.92 between the difference of the 2 means to the probable error of their difference leaves no question as to the effectiveness of gill extracts in bringing about tumor regression. Although none of the gill extract injected tumors underwent complete regression within 12 days following the first injection, 7 of the 11 cases degenerated completely by the time they were killed for autopsy or had died (Table I). None of the controls evidenced complete degeneration.

Lastly, the results of sterile water injections are listed in Table I. It is evident here that although one case showed neither a reduction nor an enlargement in tumor size, the other

4 enlarged moderately within the time limits of the experiment, so that the average increase of the 5 tumors amounted to 14.1%. The 4 control tumors experienced the usual fluctuations in size and averaged a 2.7% reduction. Distilled water, as might be expected therefore, apparently inhibits tumor growth little, if any. The ratio calculated here between the difference of the means to its probable error was 4.10, but it must be remembered that the water injected tumors increased more or less normally in size while the control injected tumors underwent slight regression.

Summary. Injection of neutralized acid extracts of histolyzing tail skin from *R. catesbeiana* larvae undergoing normal metamorphosis into spontaneous mammary tumors of mice caused regression of tumors, as reported earlier. This oncolytic substance retains some activity after 15 months at $5 \pm 2°C$. Extracts made of histolyzing *R. clamitans* tail muscle from larvae undergoing artificial metamorphosis are not oncolytic. In this respect they correspond to *R. catesbeiana* tail muscle extracts made from normally metamorphosing larvae. Extracts made from histolyzing gills of artificially metamorphosed *R. clamitans* larvae produce pronounced regression of mammary tumors. The effect of injecting sterile distilled water is practically nil, although the rate of tumor growth may be slightly retarded in some instances. It is concluded that extracts made from histolyzing larval anuran gills, similar to those made from degenerating larval anuran tail skin, contain some substance or substances which exert an inhibitory and regressive influence on spontaneous mammary tumors of mice. The results are statistically significant as compared with the mild regressive influence of control solutions similarly injected.

1. Helff, O. M., PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 336.

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Catabolism of Glucose by Mammalian Tissues. (21575)

BEN BLOOM. (Introduced by S. S. Chernick)

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In a previous communication(1) it was shown that rat diaphragm sections catabolize glucose in a manner predictable from the assumption of exclusive operation of the Embden-Meyerhof(E.M.) pathway. This finding was confirmed by the demonstration that incubation of rat diaphragm with glucose-6-C^{14} and glucose-1-C^{14} results in essentially identical yields of $C^{14}O_2$(2). With rat liver slices it was found that the sixth carbon of glucose gave rise to CO_2 in about 35% of the yield derived from the first carbon of glucose. Evaluation of the ratio of $C^{14}O_2$ formation from glucose-6-C^{14} and glucose-1-C^{14} has been made for liver slices of rats in various states(3), yeast(4), various plant materials(5), and rat mammary glands (6). It should be noted, however, that this ratio can be used as a quantitative measure of the fraction of glucose catabolized via the E. M. pathway only when it has a value close to unity. Other values of this ratio are only of qualitative significance(6,7).

In the present study the yields of $C^{14}O_2$ from glucose-1-C^{14} and glucose-6-C^{14} have been compared for several rabbit tissues.

Methods. Glucose-1-C^{14} and glucose-6-C^{14} were obtained from the National Bureau of Standards. The incubation medium consisted of Krebs-Ringer bicarbonate buffer as described by Umbreit *et al.*(8). Non-isotopic sodium gluconate, sodium lactate, and sodium acetate were included in the medium in order that the conditions might be comparable with previous studies(1,2). A description of the incubation flask, as well as other details of the incubation procedure, have been reported (1).

TABLE I. *In Vitro* Conversion of Glucose-C^{14} to $C^{14}O_2$. Rabbit tissues were incubated 3 hr at 37.8° with 5.5 ml of bicarbonate buffer containing 50 μM each of glucose, gluconate, lactate and acetate. Location of isotope in labeled glucose is indicated below. Radiochemical yields of CO_2 calculated/500 mg of tissue.

Tissue	Rabbit No.*	Radiochemical yield of CO_2 from glucose, % -1-C^{14}	-6-C^{14}	Ratio, G-6-C^{14}/ G-1-C^{14}
Brain	1	2.78	2.58	.93
	2	3.12	2.93	.94
	3	3.85	3.56	.92
	4	3.18	3.31	1.04
Kidney	1	1.38	1.52	1.10
	2	2.01	1.73	.86
	3	1.70	1.04	.61
	4	2.45	2.27	.92
Heart	2	.60	.36	.60
	3	.73	.61	.83
	4	.71	.52	.73
Spleen	1	1.88	.93	.49
	2	2.36	.83	.35
	3	2.34	1.09	.48
	4	2.06	1.18	.57
Liver	1	3.18	1.12	.36
	2	3.63	1.44	.40
	3	5.67	1.59	.28
Bone marrow	1	2.02	.62	.30
	2	1.05	.28	.27
Testis	1	2.40	.76	.32
	4	4.37	1.29	.29

* No. 1 and 2 were chinchilla rabbits, ♂ and ♀, respectively. No. 3 and 4 were white rabbits, ♀ and ♂, respectively.

Results. Recoveries of $C^{14}O_2$ upon incubation of various rabbit tissues with glucose-1-C^{14} and glucose-6-C^{14} are recorded in the Table. The ratios of the recoveries obtained with kidney and liver slices are in good agreement with those previously found with rat tissues. With rabbit brain slices a value close to unity was obtained, indicative of the unique operation of the E. M. pathway for the catabolism of glucose by this tissue. Bone marrow and testis slices converted the first carbon of glucose to CO_2 in yields of about 3 times that derived from the sixth carbon. Cardiac muscle and spleen slices yielded ratios of intermediate value.

The only catabolic pathways for glucose in mammalian tissues for which a substantial body of information exists are the E. M. and the phosphogluconate oxidation pathways (9,10). One of the characteristics of this latter pathway is the preferential appearance of carbon one of glucose as CO_2 and this requirement is satisfied by those $C^{14}O_2$ ratios less than unity. With rat liver slices a further elucidation of the nature of the non-E. M. pathway has been achieved by the demonstration that glucose-2-C^{14} and ribose-1-C^{14} behave similarly in the asymmetric labeling of carbons –1 and –6 of glycogen(11). These findings are also in accord with the mechanism of the phosphogluconate oxidation pathway.

It is of interest to note that assays of phosphogluconate dehydrogenase activity(12,13) in cell free systems of various tissues have shown that skeletal muscle is practically devoid of this enzyme and that the activities of other tissues parallel the present findings.

Summary. A comparison has been made of radiochemical yields of CO_2 from glucose-6-C^{14} and glucose-1-C^{14} on incubation with various rabbit tissues. Brain slices appear to catabolize glucose practically entirely via the E. M. pathway. The other tissues studied, particularly testis and bone marrow, formed CO_2 in greater yield from carbon-1 of glucose than from carbon-6 of glucose.

1. Bloom, B., Stetten, M. R., and Stetten, D., Jr., *J. Biol. Chem.*, 1953, v204, 681.

2. Bloom, B., and Stetten, D., Jr., *J. Am. Chem. Soc.*, 1953, v75, 5446.

3. Agranoff, B. W., Brady, R. O., and Colodzin, M., *J. Biol. Chem.*, 1954, v211, 773.

4. Beevers, H., and Gibbs, M., *Nature*, 1954, v173, 640.

5. ———, *Plant Physiol.*, 1954, v29, 322.

6. Abraham, S., Hirsch, P. F., and Chaikoff, I. L., *J. Biol. Chem.*, 1954, v211, 31.

7. Bloom, B., and Stetten, D., Jr., *ibid.*, 1955, v212, 555.

8. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., *Manometric Techniques and Tissue Metabolism*, 1949.

9. Horecker, B. L., *Brewers Digest*, 1953, v28, 214.

10. Racker, E., *Advances in Enzymol.*, 1954, v15, 141.

11. Bloom, B., Eisenberg, F., Jr., and Stetten, D., Jr., *J. Biol. Chem.*, in press.

12. Horecker, B. L., *Phosphorus Metab.*, 1951, v1, 117.

13. Glock, G. E., and McLean, P., *Biochem. J.*, 1954, v56, 171.

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